Optimising the Culture Environment for Early Juvenile Pot-bellied Seahorses *Hippocampus abdominalis* Leeson, 1827 (Teleostei: Syngnathidae)

A Thesis Submitted

in Fulfilment of the Requirements

of the Degree of

Doctor of Philosophy

School of Aquaculture

Faculty of Science, Engineering and Technology

University of Tasmania

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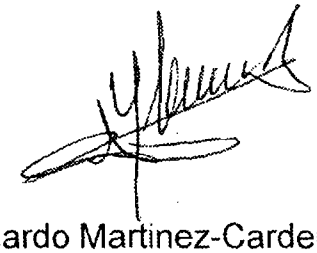
Launceston, Tasmania

2007

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the university or any other institution, except by way of background information and duly acknowledged in the thesis and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgements are made in the text of this thesis.



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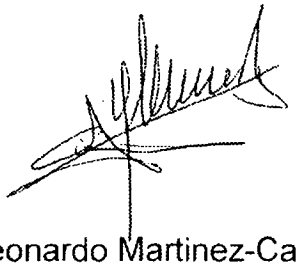

Leonardo Martinez-Cardenas

Abstract

The general aim of this study was to address questions on early juvenile *Hippocampus abdominalis* husbandry posed by research and commercial ventures. Experiments assessed the effect of tank colour, temperature, salinity, stocking density, photoperiod and substrate on survival, growth and *Artemia* ingestion of cultured juveniles.

Seahorses are visual feeders; experiments tested the effects of eight background colours (black, blue, green red, orange, yellow, white and clear) and three photoperiods on prey intake and growth. Ten minute observations and growth trials indicated no significant differences among treatments. Seahorses improved growth in 16:08 (L:D) compared to constant light and 08:16 (L:D). Seahorses under continuous light did not improve growth despite continuous feeding opportunity. A subsequent study on adults indicated that *H. abdominalis* produces elevated levels of plasma melatonin during the scotophase and low levels in the photophase.

The aquarium trade in seahorses is primarily focused on tropical species. To assess the adaptability of *H. abdominalis* to tropical conditions, the effect of four temperatures (17, 20, 23 and 26 °C) on juvenile survival and growth was investigated in two 6-week experiments. Seahorse growth was higher at 20 and 23 °C than 17 °C while 100% mortality occurred at 26 °C.

The availability of space in seahorse culture depends on the availability of the attachment substrate in addition to free tank space used during swimming and forgaing. The effect of different stocking-densities and substrate preferences on *H. abdominalis* was examined. Four stocking densities (45, 30, 15 and 5 juveniles 3 l^{-1}) were tested on newborns over six weeks. A second experiment aimed to remove the mortality effect experienced during the first experiment using 21-day-old fish to test three stocking-densities (25, 15 and 5 juveniles 3 l^{-1}). There were no significant differences between treatments in both trials. Juveniles were provided with three choices in substrate diameter (0.17, 0.55, 0.90 mm) and mesh-density (5, 10 and 24 mm in bar–length, giving high, medium and low mesh density). Newborns and 28-

day-old seahorses displayed preference for larger diameters and low mesh-density.

Commercial facilities culturing *H. abdominalis* occasionally experience reduced salinities during seasonal rainfall runoff. To determinate the adaptability of this species to low salinities, juveniles were direct- and gradual-transferred from 32 ppt to salinities down to 5 ppt. Juveniles grew and survived in a range of 32 to 15 ppt while 5 ppt produced 100 % mortality.

From the study, the environmental conditions which potentially promote optimal juvenile growth and survival are a temperature of 20 °C, a salinity of 20 ppt, a stocking density of 5 seahorses l^{-1} and a photoperiod of 16:08 (L:D). The information from this study identifies the baseline for future research protocols and provides scientific information on the husbandry of *H. abdominalis* juveniles that can be applied to commercial culture.

Acknowledgments

First and foremost I would like to thank my supervisor Dr. John Purser, for an immeasurable amount of assistance, guidance, encouragement and opportunity over these years.

Various sections of this thesis would not have been possible without the collaboration of Dr Natalie Moltschaniwskyj, Dr. Mark Porter and Dr. Mark Powell; their knowledge, assistance and guidance through the project has been greatly appreciated,

Many thanks to Murray Frith and Greg Kent for providing valuable guidance during my PhD.

Special thanks to Renee Florent for her kind assistance during experiments and thesis preparation– without Renee I would not have made it.

Also thanks to many people and organizations that have assisted me, whether it is technically, financially or otherwise over the duration of my candidature: Consejo Nacional de Ciencia y Tecnologia Mexico, Universidad Marista de Merida, Mexico, Jan Daniel, Annabel Tyson, Deborah Wagner, Ryan Longland, Detlef Planko, Matthew Flood, Andrew Bridle, Rebecca Van Gelderen, Vicky Walker, Matt Foale† and anyone else that I have forgotten to mention.

I dedicate this thesis to the memory of my mother Margarita Cardenas Ramirez.

Thanks to my family for their support throughout the years it has been without limits. I owe the warmest of thanks to my father; I would not have managed it without his guidance. Thanks to my brother Gerardo Martinez, Elizabeth Jennis and Michael Attard for their encouragement. Thanks to my friends at home for the long-distance advice.

Table of Contents

List of Figures

Figure number *Page number*

List of Tables

Table number *Page number*

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

All 33 recognized species (Lourie *et al.*, 1999) of seahorse belong to the genus *Hippocampus* a member of the family Syngnathidae which includes seadragons, pipefish and pipehorses (Foster and Vincent, 2004). Seahorses are one of the most popular marine genera in the aquarium trade worldwide, the demand being met primarily by wild-caught individuals with only a small number of seahorses cultured commercially. Cultured individuals are generally more expensive than those captured from the natural fish stock. Also the number of dead dried seahorses that are sold as a by-product of aquaculture to be used as curiosities or ornaments is smaller than the number of dead seahorses obtained from the wild to be used as curiosities (Vincent, 1995).The seahorse industry faces different challenges compared to the culture of other aquarium fish species. For instance, while other marine species accept artificial food, seahorse culture relies primarily on live food which is more expensive to culture (or capture) requiring the use of additional infrastructure to maintain the live food quality (Woods, 2003c). This characteristic restricts the seahorse market range to the experienced aquarist. Seahorses are also part of the displays of recreational parks such as Sealife (Merlin Entertainment) and public aquaria in several cities around the world such as Melbourne and Sydney in Australia and Chicago in USA. Some facilities like Seahorse World Pty. Ltd. and Ocean Rider, Inc. also provide guided tours to the public for educational purposes.

It was not until 1995 when surveys on the seahorse trade were made public world-wide that the effect of overfishing on natural populations became evident (Vincent, 1996). The Traditional Chinese medicine (TCM) uses dried seahorses as an ingredient in a number of medications to treat illness such as asthma, arteriosclerosis, incontinence or as aphrodisiacs (Vincent, 1995), and meeting this demand relies on fishing to a greater degree than aquaculture. The pressure inflicted on the natural populations by overfishing has resulted in the addition of the entire genus *Hippocampus* to the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Foster and Vincent, 2004). In addition, one species of seahorse is considered endangered, nine as vulnerable and the remainder as data deficient by the 2003 World Conservation Union

(IUCN) Red List (Lourie *et al.,* 2004). Some of the actions that have been adopted to alleviate pressure on wild populations are the establishment of natural reserves and fishing regulations with the agreement of seahorse fisheries and governments (Vincent and Pajaro, 1997). The establishment of culture techniques may also alleviate pressure on wild populations of seahorses (Shapawi and Purser, 2003).

From the 33 recognized seahorse species in the world listed in Lourie *et al.* (2004) 10 are currently bred in captivity at a commercial scale and four are found in estuaries: the pot-bellied seahorse *H. abdominalis*, yellow seahorse *H. kuda*, long snout seahorse *Hippocampus reidi* and Knysa seahorse *Hippocampus capensis*. In 1988 the latter species, was included as endangered in the Appendix II of CITES as their population declined due to mortalities caused partially by freshwater floods in the Knysna estuary despite the species' tolerance of a broad salinity range (1-59 ppt). At present, *H. capensis* is being successfully cultured and recently available to the aquarium trade (Ocean Rider, Inc., Hawaii) through the development of appropriate culture techniques.

Seahorse biology

Seahorse biology features some unique biological processes among the animal kingdom such as their reproductive strategy, which comprises a spectacular courtship display after which the female transfers the eggs to the male brood pouch, where a pregnancy takes place. In temperate water species such as *H. abdominalis*, about 300 (from 8-month-old adults) to 1000 (from 2-year-old adults) juveniles resembling adults in form and completely able to swim and feed, emerge from the male's pouch 3-4 weeks after the egg transfer. In some tropical species such as *H. zozterae* and *H. kuda* the gestation period is just two weeks (Foster and Vincent, 2004). *H. abdominalis* can breed all year round in the wild (Poortenaar *et al.*, 2004) and also in captivity where constant temperature can be maintained (Woods, 2001). The relative small numbers (compared to other teleosts) of newborns produced in each brood are the result of the specialized reproductive biology of seahorses and means that in research, limited numbers of fish will be available for experimentation.

Seahorse social interaction in their natural environment includes daily greetings as part of the courtship. This behaviour has been associated with monogamy as some species retain the same mate for prolonged periods (Kvarnemo, 2000). Vincent *et al.* (2004) suggested that the monogamous pairing in *H. whitei* benefits the species reproduction by: 1) the reduction of the differences in the potential reproductive rates of both sexes; and 2) by the introduction of a familiarity effect due to successive mating. This characteristic represents a disadvantage when these patterns are disrupted by the capture of one of the mates (Foster and Vincent, 2004). The monitoring of pairing behaviour, growth and mortality has been improved by the use of successful tagging techniques in adults such as implants of fluorescent elastomer (Woods and Martin-Smith, 2004) and passive integrated transponders (PIT) tags (Woods, 2005). However, there is nothing in the scientific literature demonstrating the use of any device to successfully tag early seahorses.

Seahorses have a similar basic body morphology constituted by a right-angled horse-like head, stretched skin over a series of cartilaginous rings (Gomon and Neira, 1998; Kuiter, 2000). Unlike other teleosts, seahorses have a prehensile tail and use it in a postural role as a grasping and holding appendage (Hale, 1996). During seahorse evolution the tail ceased playing any locomotor role in swimming. However in newborns of *Hippocampus kuda* and *Hippocampus mohnikei* vestigial caudal rays are present at the tail tip but these vanish 3-4 days after birth (Choo and Liew (2006). Seahorse temporarily hold onto substrates such as sponge, branching coral, seagrass or submerged tree branches in their natural habitat, and also onto artificial structures such as fishnets and cages (Wilson and Vincent, 1998). During periods of inactivity (mostly during the scotophase or dark phase) seahorses tend to hold onto substrates more than during the photophase (Ouyang, 2005). Most seahorse species grasp on substrate as part of their ambush strategy while preying primarily on live, mobile prey types. In the wild *H. abdominalis* feeds primarily on crustaceans (Woods, 2002), while in captivity they accept copepods and *Artemia* nauplii in the early stages and frozen mysids approximately four months after birth.

Most seahorse species are distributed in coastal habitats in shallow temperate and tropical waters (Foster and Vincent, 2004). The pot-bellied seahorse is a coastal and estuarine species found in temperate coastal waters of southeast Australia and New Zealand (Gomon and

Neira, 1998; Martin-Smith and Vincent, 2005). This species experiences a temperature range of 8–24 °C in the wild (Woods, 2001) while a temperature range of 10-19 °C has been used in captivity (Woods, 2000b). It is also one of the largest species of seahorse in the world, with a maximum recorded length (distance between the tip of the coronet to the tip of the uncurled tail) of 35 cm (Foster and Vincent, 2004). The pot-bellied seahorse is capable of breeding all year round (Poortenaar *et al.,* 2004) though, environmental parameters such as temperature and photoperiod can influence the breeding season (Woods, 2000b). Seahorse density in their natural environment tends to be low. *H. abdominalis* is one of the species with the lowest mean densities recorded of 0.007 animals per m^{-2} (Foster and Vincent, 2004). However, commercial scale *H. abdominalis* culture has been successfully conducted at a maximum stocking density of 100 adults in 1000-l tanks and juveniles have been reared at stocking densities of 28, 000 fish in 2 m^3 Rathburn tanks (Seahorse Australia; Purser, G.J. pers. comm.).

Seahorse aquaculture and research

Early research projects on the pot-bellied seahorse have addressed their feeding, mating and locomotion behaviour (Lovett, 1969). This information increased the interest in their culture at the beginning of the 1990's when a number of studies on seahorse husbandry were conducted (Fam, 1992; Scarratt, 1996). However, those efforts lacked consistency in the methods used (i.e. different age/size of the seahorse examined) leaving gaps in the knowledge of their culture. During the late 1990's commercial seahorse culture to some extent was conducted using *ad hoc* practices, which despite their functionality, suggested a need for the development of better culture technology based on scientific experimentation. For this reason research links were formed between research organizations and commercial facilities (e.g. University of Tasmania and Seahorse World Pty. Ltd. Tasmania) to better facilitate the experimentation.

Investigations into seahorse culture, including *H. abdominalis,* have been conducted primarily on feeding and husbandry of late juveniles as early stages present an inherent higher mortality rate and their small size increases the difficulty of collecting the minimum quantities of body samples for analysis and comparison. Payne and Rippingale (2000)

conducted studies on *Hippocampus subelongatus* feeding; the authors found that survival of early juveniles was better in copepod-fed fish than juveniles fed exclusively with enriched *Artemia*. In the same year Woods (2000b) conducted preliminary research on seahorse husbandry in which reported that the use of an *Artemia* dominated diet could be less effective than a diet comprised of a variety species such as amphipods (e.g., caprellid and ischrocerid amphipods), caridean shrimp (i.e. *Hippolyte bifidirostris*) and pericarids (i.e. the mysid *Tenagomysis similis*) (Woods 2002). In 2003 Woods produced two studies dedicated to the determination of the optimal feeding condition for *H. abdominalis*. Woods (2003c) tested the alternative use of frozen mysids instead of live *Artemia* on *H. abdominalis* late juveniles and found that although no advantages in growth were found, frozen mysids are highly recommended for fish sold into the aquarium trade for their convenience. Woods (2003b) found that the more expensive *Artemia* enrichment does not necessarily produce the best seahorse growth as the use of a mixture of non-expensive enrichment and *Spirulina* produced similar results to the more expensive commercial enrichments such as Super Selco®.

At the beginning of the present study in 2003 limited information was available on *H. abdominalis* husbandry. The few studies published did not always provide a detailed description of the techniques used or the characteristics of the fish observed. That was the case of the study published by Woods (2000a) on early juvenile husbandry on which Chapter Two of this thesis was based. The chapter provides a detailed description of the age/size used, a wider range of the variables tested, and the use of more extensive statistical analysis of the results compared to the study of Woods (2000a).

The number of published research papers focusing on pot-bellied seahorse increased during the course of this study. Some of these publications have approached topics similar to those examined in the present work. The project is also part of the seahorse research program conducted by the School of Aquaculture since 1993, which has produced studies such as: Thomson (1999), Adams *et al.* (2001), Leef (2001), Wardley (2001), Ouyang (2005) and Wilson *et al.* (2006).

In the aquarium trade there is greater demand for intense skin colours (i.e. red, yellow, orange), which are more readily found in tropical seahorse species such as *Hippocampus erectus, Hippocampus ingens,* and *Hippocampus kuda.* Seahorse colouration can also be affected by animal interactions such as mating (Vincent and Sadler, 1995). Research to determine the effect of background colour on the skin pigmentation of late juvenile *H. abdominalis* has found that skin colouration changes take place over a longer period of time (Wardley, 2001) compared to tropical species. That study showed that the skin colour of *H. abdominalis* can change in response to background colour, particularly yellow and white.

Tank colour is an important factor to consider in the culture of fish, as different coloured backgrounds can induce a variety of responses such as food intake, growth, survival and stress (Gleyzer, 1983; Gilham and Baker, 1985; Moriya and Miyashita, 1987; Papoutsoglou *et al.*, 2000; Tamazouzt *et al.,* 2000). A preliminary observation on the effect of background colour on *Artemia* ingestion of early juvenile *H. abdominalis* seahorses was conducted by Woods (2000a). In that study a positive feeding response of newborns in transparent tanks compared to the *Artemia* of juveniles in black tanks was attributed to the better contrast of the darkened guts of the algae-enriched nauplii against the clear tank walls. However, no study has been conducted on the feeding response of seahorses to the visual contrast of their prey against background colours such as blue, green, yellow, orange, red or white. As seahorses are visual feeders, it is important to provide conditions to optimize prey ingestion to maximize growth and survival. Additional factors which may influence this process include light and photoperiod (Boeuf and Le Bail, 1999).

There is considerable information regarding the effect of light intensity and photoperiod that can alter sexual behaviour, food intake and activity patterns in fish over a 24 h cycle (Reebs, 2002). The commercial culture of *H. abdominalis* is generally conducted within a range of 12-13 h of light in order to provide similar conditions to those in nature (Seahorse World Pty. Ltd. pers. comm.). A preliminary study conducted by Woods (2000b) reported that an increase of the photophase above 11 h promoted courtship behaviour in cultured *H. abdominalis* while a reduction from 11 h inhibits it, suggesting that mating is partially driven by day length. During a histological analysis of wild female *H. abdominalis,* Poortenaar *et al.* (2004) found reproductively mature seahorses all year round. This means that while day

length may influence mating and courtship, seahorses appear to have the capacity to reproduce year-round. Research is needed to determine the more specific effects of photoperiod manipulation on the life history of this species.

Woods (2000a) found positive phototaxis and improved growth and survival in early juveniles *H. abdominalis* in relation to light orientation. The light attracted the fish to the side of the tanks, preventing the access to the surface, which can lead to swim bladder hyperinflation. A study conducted by Florent (2003) also on early juveniles, tested a similar design to Woods (2000a), and confirmed that swim bladder hyperinflation can be affected by light orientation. Literature studies regarding feeding activity of cultured seahorse have reported low activity during the dark period (Karina *et al.*, 2006; Sheng *et al.*, 2006). Similarly Ouyang (2005) found that late juvenile *H. abdominalis* displays activity primarily during the photophase. However, the effect on growth and survival of early juvenile seahorses exposed to different photoperiods has not been reported.

Furthermore, in most vertebrates (fish included) light changes are processed by the pineal organ and result in hormonal signals such as melatonin, which has been associated with the mediation of physiological processes (Pavlidis *et al.,* 1999). Melatonin also plays a role in the transduction of light information to reproductive and growth processes in fish (Bayarri *et al.*, 2004). Reiter (1989) defined the three main melatonin synthesis profiles: type A which presents a low melatonin level during the light phase and an increase towards the end of the scotophase; type B which maintains low levels under the light period and the production of melatonin increases to reach the maximum point in the middle of the dark phase; type C characterised by a prolonged peak during most of the scotophase. The third type is the most frequent melatonin production pattern in fish. The commercial culture of marine fish species such as Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua* has benefited from the determination of melatonin production patterns (Randall *et al.,* 1995; Porter *et al.,* 2000); with the use of photoperiod manipulation, the salmon industry has prevented maturation of fish before they reached harvestable size (Porter *et al.,* 1999). There is no record in the literature of melatonin production in seahorses. Hence, the determination of a melatonin profile in seahorses and the impacts of the different photoperiods on melatonin production could benefit the seahorse industry through the manipulation of growth and reproduction.

One of the most important factors that the aquaculture industry has employed to optimize fish culture is temperature (Tucker, 1998). While the aquarium industry prefers warm-water species of seahorses, which are more compatible with tropical fish tanks, there is a limited trade in the two commercially cultured temperate-water seahorses in the world: the pot-bellied seahorse *H. abdominalis* and the short-snouted seahorse *Hippocampus breviceps* (Seahorse World Pty. Ltd.; South Australian Seahorse Marine Services; Seahorse Australia Pty. Ltd.). White's seahorse *Hippocampus whitei* is also cultured for the aquarium trade at temperatures lower than tropical but higher than those used in temperate seahorses based on the temperature ranges experienced in their natural distribution (Wong and Benzie, 2003). The authors reported improved growth at temperatures up to 26 °C, but declines in the condition index suggested an optimal temperature of 20°C for the culture of that species. Woods (2001) reported improved growth of late juveniles of *H. abdominalis* cultured in temperatures up to 21 °C. In that study the author provided little detail on the culture techniques used (i. e. temperature acclimation prior to experiments, feeding rate). Both studies provided valuable information on the culture of this species. However, both were focused on late stage seahorses and neither provided information on the effect of different temperatures on early juveniles.

The interaction of water temperature and salinity has improved growth in some commercially cultured species. Early turbot *Scopthalmus maximus* juveniles cultured in low salinities (15 ppt) combined with higher temperatures (23 °C) improved growth and food conversion efficiency (Imsland *et al.*, 2001). In a laboratory study conducted by Hilomen-Garcia *et al.* (2003) the tropical seahorse *H. kuda*, tolerated a salinity range of 10-50 ppt over the short-term (18 days). In addition, the authors recommended the culture of that species in brackish water as they found improved growth and survival when cultured in 15-20 ppt. Seahorse culture in Tasmania experiences seasonal fluctuations in salinity due to runoff of rainwater into estuaries and coastal waters, decreasing the surface salinity to 15 ppt (Seahorse World Pty. Ltd. per. comm.). However, information is lacking on the effect of reduced salinities on *H. abdominalis* growth and survival.

In Tasmania, the interaction of temperature and salinity in the natural environment of *H. abdominalis* occurs generally in the winter season when fish can be exposed to water

temperatures as cold as 8 °C and salinities as low as 15 ppt. In the summer the interaction salinity-temperature is generally between temperatures as high as 19 °C and salinities of 32-35 ppt. However, during the summer season the seahorse industry is affected by another husbandry issue which is the determination of an optimal stocking density. The warmer temperatures of summer generate breeding peaks during which the holding capacity of the rearing systems is often exceeded (Seahorse World Pty. Ltd. pers. comm.). Compared to more r-selected marine teleosts that produce small larvae in great numbers, the genus *Hippocampus* gives birth to small broods of well-developed fish due to their more K-selected reproductive strategy. Newborn seahorses, which are considered juveniles, are cultured at relative lower stocking densities than species with pelagic eggs, but at a higher density compared to seahorses under natural conditions. The utilization of suboptimal stocking densities can compromise water quality in fish culture systems. Ammonia levels produced from the catabolism of dietary and structural proteins increase oxygen consumption by seahorses with mortalities occurring at total ammonia nitrogen concentrations of 14.8 mg l^{-1} (Adams, 2001). Wong and Benzie (2003) did not find a major effect on *H. whitei* growth or survival at a stocking density of 1 seahorse l^{-1} compared to 0.5 seahorse l^{-1} although some indication of reproduction inhibition was recorded at a stocking density of 1 seahorse l^{-1}. Woods (2003a) tested 1, 2 and 5 juveniles l^{-1} on *H. abdominalis*, and found decreased growth of seahorses at 5 juveniles l^{-1}. Although those studies provided useful information for the seahorse industry they were focused on late juveniles, testing a limited number of seahorses per litre. During the commercial culture of *H. abdominalis* in Tasmania, Seahorse World Pty. Ltd., allocates approximately 400 juveniles which can be a combination of two broods, to 50-l tanks (8 seahorses l^{-1}). Although this practice has been relatively successful, the optimal density for early stages or the effect of higher densities on juveniles has not to been determined scientifically.

While in other teleost species the determination of an optimal stocking density relies on water volumes, in seahorse culture the concept of living space applies differently. In seahorse culture the space availability during periods of inactivity and during the scotophase depends on the suitability of the substrate as fish tend to hold with their tail rather than swim continuously (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006). Seahorse culture in captivity has used a diversity of materials to provide attachment substratum: artificial plants,

plastic mesh and filaments. In general, early seahorse stages are predominantly pelagic becoming benthic after 2-3 weeks (Choo and Liew, 2006). *H. abdominalis* is considered pelagic during the first month of its life cycle (Gomon and Neira, 1998). However, it is noted by the author that even 2-day-old juveniles used the attachment substratum. The seahorse industry requires more research on cost-effective husbandry solutions to seahorse requirements. The preference of substrate diameter and density by the fish may determine the activity level and the distribution of fish within the culture tanks.

This study was developed to address a number of questions posed by previous researchers and commercial operators. This included: does early juvenile *H. abdominalis* grow better in tank of a specific colour? Can early juvenile *H. abdominalis* tolerate tropical conditions? During the peak of the breeding season, how many seahorses per litre can be cultured? What is the lower salinity tolerance, which the industry should be aware of? Will seahorses benefit from a continuous light/feeding regime? Are they indiscriminant in their attachment-substrate choice? Therefore the following aims have been proposed.

- To determine which tank (background) colour promotes the best *Artemia* ingestion, survival and growth (Chapter Two);
- To examine the potential of culturing early juveniles in temperature conditions close to those used by the tropical aquarium trade and the effect of those temperatures on *Artemia* ingestion, survival, growth, moisture content and the C:N ratio. As an additional aim, the response of juveniles to different acclimation periods will be examined (Chapter Three);
- To evaluate the effect of culturing early juveniles at high stocking densities on *Artemia* ingestion, survival and growth (Chapter Four);
- To determine the tolerance of early juveniles to water salinities lower than 32 ppt and the effect on *Artemia* ingestion, survival growth, sodium: potassium ratio and whole body osmolality (Chapter Five);
- To investigate the effect of extended/reduced photoperiods in early juveniles on *Artemia* ingestion, survival and growth (Chapter Six);
- To determine the existence of melatonin in seahorses and its production profile over

24 h (Chapter Seven);

- To examine if early juveniles exhibit a preference for a specific substrate size, or if they are indiscriminant in their selection (Chapter Eight).

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CHAPTER 2

EFFECT OF TANK COLOUR ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

This chapter has been published as:
Martinez-Cardenas L., Purser G. J., 2007. Effect of tank colour on *Artemia* ingestion, growth and survival in cultured early juvenile pot-bellied seahorses (*Hippocampus abdominalis*). Aquaculture 264, 92-100.

2 EFFECT OF TANK COLOUR ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

2.1 Abstract

Seahorses are visual feeders and as such it is important to provide conditions to optimise visual acuity enabling fish to detect and ingest prey effectively to maximise growth and survival. The effect of the background (tank) colour on *Artemia* ingestion, growth and survival of early juvenile seahorses *Hippocampus abdominalis* was investigated in two experiments. In short-term trials, eight tank colours (clear, white, yellow, orange, green, red, blue, black) were used to quantify the *Artemia* ingestion in 42-day-old fish, and 7-day-old fish. No statistical difference was observed among treatments. In the second experiment 3-day-old seahorses were cultured over six weeks in one of six coloured tanks (clear, white, yellow, red, blue, black) in a temperature and photoperiod controlled recirculation system to determine the effect of the background colour on growth, survival and conditioning to tank colour. At the end of the experiment there were no significant differences as early juvenile seahorses were able to feed, grow and survive in any of the tank colours tested. Seahorses did not appear to display any conditioning to the tank colour in which they were cultured.

Keywords: *Static tanks; recirculation system; feeding activity; feeding efficiency; colour conditioning*

2.2 Introduction

Early stage teleost fish are typically visual feeders and as such it is important to provide conditions to optimise visual acuity enabling fish to detect and ingest prey effectively to maximise growth and survival. Conditions which may influence this process include prey density, orientation of lights, light intensity and wavelength, contrast of prey against the background, tank colour, water turbidity and prey colour (Denderinos *et al.,* 1984; Duray *et al.,* 1996; Naas *et al.*, 1996; Downing and Litvak, 1999).

Tank colour is an important factor to consider in the culture of fish, as different coloured backgrounds can induce a variety of responses relative to food intake, growth, survival and stress (Gleyzer, 1983; Gilham and Baker, 1985; Moriya and Miyashita, 1987; Papoutsoglou *et al.*, 2000; Tamazouzt *et al.,* 2000). Several fish species prefer dark tank walls (Ostrowski, 1989; Naas *et al.,* 1996) as they promote a suitable contrast between the prey and the background colour (Browman and Marcotte, 1987). However, some studies have shown a preference by some fish species for light tank colours (Papoutsoglou *et al.*, 2000; Tamazouzt *et al.*, 2000).

Seahorses are visual predators, and as they are not high-speed swimmers they adopt a sit and wait ambush strategy (Lovett, 1969), combined with a distinctive rapid suction feeding mechanism (Bergert and Wainwright, 1997). Juveniles are able to feed on the day of release from the pouch of the male. Typically experimentation in seahorse husbandry has been conducted predominantly on fish older than two or three months of age (Hilomen-Garcia *et al.*, 2003; Wong and Benzie, 2003; Woods, 2003b; c), with very limited work undertaken on the early development stages in the first few weeks.

The pot-bellied seahorse *Hippocampus abdominalis* has been cultured for a number of years in Australia and New Zealand principally for the aquarium trade. Typically the species is reared on enriched *Artemia* instar I-III in the first few weeks and then weaned onto larger *Artemia*, mysids or amphipods. While studies have reported on aspects of its feeding and growth (Woods, 2000; 2003c), limited information is available on the effect of tank colour on its survival and growth. In a study by Woods (2000) 7-day-old *H.*

abdominalis juveniles displayed the highest prey attack rate in clear tanks followed by black tanks and white tanks. However this preference was less evident when the fish were tested at 30 days-old.

The present study builds on the design of Woods (2000) by testing prey ingestion and growth in similar aged *H. abdominalis* under similar environmental conditions but over a greater range of tank colours; eight colours were tested in short term trials and six in longer term trials. Such an extensive range of colours is not usual in marine fish testing but there is some interest in using tank colour not only to optimise *Artemia* ingestion and growth, but also as an environmental stimulus to change skin colour in pot-bellied seahorses. While the aquarium industry prefers warm-water species, as they are more compatible with tropical fish tanks, a limited trade in temperate seahorse species does exist (Seahorse World Pty. Ltd.; South Australian Seahorse Marine Services; Seahorse Australia Pty. Ltd.) (Martin-Smith and Vincent, 2006). *H. abdominalis* also has been a useful seahorse model for aquaculture and biological research.

The overall aim of this study was to determine the effect of a wide range of background colours on prey ingestion, growth and survival of early juveniles of the seahorse *H. abdominalis*. Initially the experiments tested the ability of seahorses to ingest *Artemia* following transfer from a holding tank colour to one of a range of colours and in doing so tested the suitability of using prey strikes as a convenient visual measurement of food intake. Secondly the study assessed the long term suitability of a range of tank colours by measuring prey intake, growth and survival of seahorses over a six week period, followed by an evaluation of whether fish are conditioned to a particular colour during the culture period.

2.3 Materials and Methods

2.3.1 System design and general methods

Juvenile seahorses used in this study were transported three days after birth in 32 ppt (g l^{-1}) seawater and oxygen-filled plastic bags inside an insulated container from a commercial seahorse facility (Seahorse World Pty. Ltd. Beauty Point) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. The age of the seahorses used in this study was expressed as days-old, the moment of the release of the juveniles from the male's pouch was considered the moment of birth on day 0. After 15 min temperature acclimation the fish were allocated to 20-l natural (fawn) coloured fibreglass holding tanks until the start of the experiments (Appendix 1, diagram one). For short-term observations individual static tanks were used and for a long-term trial, 18 3-l tanks connected to a 100-l recirculation system were utilised (appendix one, diagram two). The recirculation system included a biofilter comprised of two stacked 40-l plastic containers. The upper container was filled with 40-mm bio balls and its floor area perforated every five centimetres to allow the outflow water from the tanks to trickle down to the container below. This lower container was used as a water reservoir in which was installed a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) that provided an inflow of approximately 2.5 l hr^{-1} $tank^{-1}$ of 20µm-filtered seawater. Attachment substratum for the fish was provided by a weighted bundle of 55 nylon monofilament segments with a length of 139.21 mm ±1.51 mm (mean ± 1 S.E.). During the 6-week trial the tanks were inspected daily for mortalities and any excess food and faeces were siphoned to waste. A photoperiod of 12:12 (L:D) (lights on at 08:00 h, lights off 20:00 h) and light intensity of 4.8 µE s^{-1} m^{-2} at the water surface were used during the experiment while water quality was maintained as follows: water temperature 17.2 °C (17.1-17.5 °C), pH 7.9 (7.8-8.0), salinity 34.5 ppt (32-36 ppt), dissolved oxygen > 75 %, total ammonia nitrogen (TAN) < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1} and nitrate < 5 mg l^{-1}. For the determination of pH, TAN, nitrite and nitrate, a colorimetric saltwater liquid test kit (Aquarium Pharmaceuticals Inc.) was used. Salinity and temperature were monitored every 24 h while TAN, pH, nitrite and nitrate were recorded every 48 h during the experiment. Initial and final length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a submerged plastic-covered 1 mm scaled sheet. Initial

and final wet-weight of seahorses were measured on an analytical balance and recorded to the nearest 0.0001g.

2.3.2 Effect of tank colour (white, yellow, orange, red, green, blue and black) on short-term *Artemia* ingestion by juvenile *H. abdominalis*

The objective of this experiment was to determine if the number of feeding strikes and the success of the feeding strikes by 7 and 28-day-old juvenile seahorses is directly affected by different tank colours in the short-term (10 min observations).

42-day-old juveniles

Fifteen 42-day-old juveniles (mean ± 1 S.E. length = 52.8 ± 0.8 mm, mean wet weight = 269.4 ± 13.5 mg) from a single brood were placed into fifteen 3-l (1 fish $tank^{-1}$) transparent tanks containing 20µm-filtered seawater. Two sets of tank colours were tested in the first trial. One set included four colours (white, red, blue and black) which were wrapped around the plastic tanks (3 tanks per colour). Another three tanks were used without wrapping (transparent) as a reference colour. The other set of colours (white, yellow, orange and green) was also wrapped around the plastic tanks (3 tanks per colour) and included three tanks without wrapping (transparent) as a reference colour. Fifteen 42-day-old juveniles (mean ± 1 S.E. length = 41.4 ± 0.6 mm, mean wet weight 127.3± 6.0 mg) from a single brood but a different batch to trial one, were place in each tank and observed following the protocol previously described for the first trial.

The fish were purged for 24 h prior to the experiment, in a 20-l holding tank. Juveniles were randomly distributed to tanks (1 fish $tank^{-1}$) and left for 30 min. One hundred live *Artemia* nauplii (mean length = 570 µm) were counted and distributed to each tank. In this study *Artemia* were 24 h enriched with Super Selco®. Continuous aeration was provided by flexible plastic tubing, ending with a 4-l hr^{-1} plastic water-dripper (Neta®) to disperse the *Artemia* throughout the water column in the tank. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the

fish. Immediately after the addition of the *Artemia*, the number of feeding strikes per min (attack rate) over a period of 10 min was recorded for each juvenile, after which each fish was measured (length and weight). This procedure was repeated three times, one every second day (3 tanks x 5 colours x 3 days). After each observation the fish were pooled together back to the holding tank. The fish used in further observations were randomly selected from the same batch.

An orthogonal analysis of variance (ANOVA, SPSS 11.5) was used to compare numbers of feeding strikes and compare fish size (weight and length) among treatments of the three days (as orthogonal factor). A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance.

7-day-old juveniles

The objective of this trial was to determine the consistency of the number and the success of feeding strikes on 7-day-old juvenile seahorses compared to 42-day-old juveniles, in different colour tanks. A one-day observation was conducted on 7-day-old juveniles (mean ± 1 S.E. length = 21.8 ± 0.2 mm, mean ± 1 S.E. wet weight = 20.5 ± 0.8 mg) from a single brood. Seahorses were placed into 15 3-l tanks (1 fish $tank^{-1}$) wrapped with the first set of four colours: white, red, blue, black and transparent (3 tanks per colour). The protocol previously described for the observations of 42-day-old juveniles was used in this trial, except that the observations were taken over a 5-min period instead of 10 min. A one-way analysis of variance (ANOVA, SPSS 11.5) was used to compare feeding strikes recorded for each treatment ($P < 0.05$). Levene's Test and residual plots were used to test homogeneity of variance.

Efficiency Index

To determine the reliability of using feeding strikes as an accurate measurement of *Artemia* ingestion, an efficiency index was calculated by the equation:
Efficiency index = [No. *Artemia* recovered (expected) / No. *Artemia* recovered (actual)],

where: No. *Artemia* recovered (expected) = No. added to the tank – No. strikes

if number < 1, there were a number of unsuccessful strikes;

if number = 1, then number of strikes = number of *Artemia* ingested;

if number > 1, then more than 1 *Artemia* ingested in some strikes, or strike missed by the observer.

One hundred live *Artemia* were added to each tank and fish allowed to feed (as described in section 2.3.2.1). The *Artemia* remaining in the tanks were recovered by sieving the contents of the tank over a 112 μm screen, washing the *Artemia* into a petri dish and counting the *Artemia* over a bright light after the addition of formalin. The actual number of *Artemia* was compared with the expected number, using the above equation.

The efficiency index was calculated using the first set of colours from the first experiment over the three-day observation and on a one-day observation of 7-day-old juvenile seahorses. The efficiency index provided a level of confidence in using the number of strikes to assess intake in two age groups of seahorses used in the short-term intake trials and equivalent to the initial and final stages of the long-term growth trial.

An orthogonal ANOVA (SPSS 11.5) was used to compare numbers of feeding strikes among treatments over the three sample days (as orthogonal factor) in the first experiment. In the second efficiency test conducted on a single day, a one-way ANOVA (SPSS 11.5) was used. In both analyses a significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance; data did not require any transformation. A Student's t-distribution test was conducted on all the treatments for all sample days in both experiments to determine whether each mean value was different from the optimum efficiency of 1.0.

2.3.3 Effect of tank colour on growth, survival and *Artemia* ingestion by juvenile *H. abdominalis* in a 6-week trial

The aim of this experiment was to assess the effect of tank colour on early juvenile seahorse survival, growth and *Artemia* ingestion over a 6-week period. Fifteen 3-day-old juveniles

from a single brood were placed into each of 18 3-l transparent tanks, after the length and wet weight of individual fish were recorded on day zero. Five colours (white, black, red, yellow and blue) were wrapped around the tanks (3 tanks per colour, randomly distributed) with an additional three clear tanks as a reference. Attachment substratum was provided as described in section one. The fish were fed daily with live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % initial body weight per day (BW d^{-1}) (dry weight *Artemia*: wet weight fish) that was divided into two equal sized meals. Feeding was at 10:00 and 16:00 h, and was maintained without any modification to compensate for growth or mortalities during the experiment. On all days it was checked there was excess food present at the end of photoperiod. Screens (150 µm) over the outlet of the tanks prevented the loss of *Artemia* during the day. Feeding time was considered from 08:00 to 19:00 daily, at the end of this period the screens were replaced with 500 µm screens to flush out the remaining unenriched *Artemia* overnight. Fish mortalities were removed on a daily basis and recorded. After 6 weeks the weight and length of the surviving seahorses were measured. Mean specific growth rate (SGR) of seahorses in each tank was calculated by: SGR % day^{-1} = $[(\ln W_f - \ln W_i)/t] \times 100$, where W_f = final weight, W_i = initial wet weight, and t = number of days.

A one-way ANOVA (SPSS 11.5) was used to compare the means of initial length, final length (mm), initial weight, final wet weight (mg), and final survival among treatments. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance.

Effect of tank colour on Artemia ingestion

Direct visual observations of the number of feeding strikes as a measurement of *Artemia* ingestion were undertaken during the long-term experiment. *Artemia* ingestion was recorded by selecting randomly a single fish per tank and counting the feeding strikes produced during a 3-min period, one minute after the food was introduced into the tank. Counting was undertaken on one seahorse $tank^{-1}$ day^{-1} (18 tanks day^{-1}) for three consecutive days in each of weeks one, three and five of the growth trial.

An orthogonal ANOVA (SPSS 11.5) was used to compare numbers of feeding strikes among treatments over the three days (as orthogonal factor) on each sample week using a significance level of $P < 0.05$. Levene's Test and residual plots were used to test homogeneity of variance.

Colour conditioning

On completion of the experiment, a condition test was conducted to determine if the fish that were cultured for 6 weeks in a specific background colour, showed a preference for that colour. A perspex cylinder (60 cm length x 7.5 cm diameter) capped at each end and filled with water was wrapped with the six tank colours (clear, white, red, yellow, blue, black) in identical proportions leaving a clear stripe at the top to allow light (4.8 $\mu E\ s^{-1}\ m^{-2}$) to enter and to observe the fish. The fish remaining in each tank were transferred (one tank at the time) to the cylinder and, were evenly dispersed along the cylinder (placed horizontally). After 5 and 10-min periods the number of fish distributed over each coloured area was recorded and data analysed using a χ^2 - independency test ($P < 0.05$).

2.4 Results

2.4.1 Effect of tank colour (white, yellow, orange, red, green, blue and black) on short-term *Artemia* ingestion by juvenile *H. abdominalis*

Effect of white, red, blue and black on juvenile H. abdominalis

42-day-old juveniles

Artemia ingestion was variable among the three-day observations ($F_{8,30} = 7.439$, $P < 0.001$)(Table 2.4-1). There were no significant differences in fish length among treatments on any observation day ($F_{8,30} = 1.712$, $P = 0.136$). The orthogonal ANOVA detected a significant interaction between fish weight and *Artemia* ingestion ($F_{8,30} = 2.816$, $P = 0.019$). An analysis of covariance was conducted and the results showed that the differences in feeding intake were not caused by the differences in weight ($F_{1,29} = 0.425$, $P = 0.520$).

Although there were differences in *Artemia* ingestion among treatments on individual days the pattern was inconsistent over the three days ($F_{8,30} = 7.439$, $P < 0.001$)(Table 2.4-1).

Table 2.4-1 Length, wet weight and feeding strikes (mean ± 1 S.E. of 3 fish day^{-1} colour^{-1}) of 42-day-old *H. abdominalis* on the three sample days in five different tank colours. Feeding strikes were recorded from one fish per tank over a 10-min period.

	Length (mm)			Weight (mg)			No. strikes		
Tank colour	Sample one	Sample two	Sample three	Sample one	Sample two	Sample three	Sample one	Sample two	Sample three
Clear	49.0±0.7	52.0±0.3	54.0±0.0	236.2±19.9	233.0±16.0	242.1±4.7	23.3±7.3	6.3±3.5	13.0±4.5
White	51.0±2.1	50.0±3.6	56.0±0.3	250.7±13.7	203.3±34.9	351.2±13.4	0.7±0.3	0.0±0.0	53.7±9.9
Red	54.0±3.1	56.0±2.1	55.0±0.6	278.3±38.2	313.2±20.4	301.8±26.2	24.3±4.5	12.7±4.1	10.7±10.7
Blue	46.0±2.2	56.0±2.1	57.0±1.7	178.1±21.1	287.6±36.9	368.1±20.2	3.7±3.2	1.7±1.7	1.0±0.6
Black	49.0±2.0	52.0±1.0	56.0±1.5	226.5±25.9	277.9±34.9	293.0±43.7	19.3±7.7	2.0±2.0	16.0±6.2

Notes: Values in samples 1-3 were analysed together.
The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P > 0.05$).

7-day-old juveniles

There were no significant differences in *Artemia* ingestion among treatments on the single observation day ($F_{4,10} = 2.317$, $P = 0.128$) (Table 2.4-2).

Table 2.4-2 Efficiency index (Expected No. *Artemia* recovered*/Actual No. *Artemia* recovered), (mean ± 1 S.E.) over three sample days in the first experiment (42-day-old seahorses).

Colour	Efficiency index one			Efficiency index two	No. strikes
	Sample one	Sample Two	Sample three	Sample (single)	Sample (single)
Clear	0.98±0.03	0.99±0.01	0.98±0.01	1.00±0.01	6.00±2.64
White	1.01±0.01	1.01±0.00	0.99±0.22	1.02±0.01	1.00±0.57
Red	0.90±0.03	1.00±0.04	1.03±0.03	1.00±0.03	17.00±4.72
Blue	1.02±0.02	1.02±0.02	1.16±0.16	1.01±0.03	5.33±2.66
Black	0.95±0.03	1.01±0.00	1.01±0.05	1.05±0.05	9.33±6.33

Notes: Values in samples 1-3 were analysed together by an orthogonal ANOVA.
The efficiency values and number of feeding strikes (mean ± 1 S.E. of 3 fish colour^{-1}) from a single day observation (7-day-old seahorses) were compared by a one-way-ANOVA. The use of superscripts has been omitted as no significant differences were found in either experiment ($P > 0.05$).

Effect of white, yellow, orange and green on Artemia ingestion by 42-day-old juveniles

There were no significant differences among treatments in fish length ($F_{8,30} = 0.397$, $P = 0.914$); weight ($F_{8,30} = 0.266$, $P = 0.972$) or feeding strikes ($F_{8,30} = 0.616$, $P = 0.758$) during the three-day observation (Table 2.4-3).

Table 2.4-3 Length, wet weight and number of feeding strikes (mean ± 1 S.E. of 3 fish day^{-1} colour^{-1}) of 42-day-old *H. abdominalis* on the three sample days in five different tank colours. Feeding strikes were recorded from one fish over a 10-min period.

Colour	Length (mm)			Weight (mg)			No. strikes		
	Sample one	Sample two	Sample three	Sample One	Sample two	Sample three	Sample one	Sample two	Sample three
Clear	39.0±0.9	43.0±1.0	39.0±2.3	103.1±18.3	150.4±11.5	111.5±11.0	2.3±2.3	0.7± 0.7	0.3±0.3
White	39.0±1.5	42.0±2.6	41.0±2.0	107.4±17.5	139.7±25.1	119.5±16.2	2.3±2.3	0.0±0.0	8.0±8.0
Yellow	40.0±1.00	43.0±0.6	41.0±1.5	95.9±17.1	137.1±4.4	114.1±11.1	5.3±5.3	1.3±1.3	3.3±3.3
Orange	38.0±1.9	45.0±0.3	43.0±2.7	104.4±11.4	171.5±18.3	131.3±32.7	8.0±1.0	2.7±2.7	5.7±3.2
Green	39.0±2.7	45.0±0.9	44.0±0.7	114.3±21.9	159.1±5.3	149.6±3.3	0.0±0.0	3.7±1.9	3.7±3.7

Notes: Values in samples 1-3 were analysed together by an orthogonal ANOVA.
The use of superscripts has been omitted as no significant differences were found among treatments ($P > 0.05$).

Efficiency Index

42-day-old juveniles

The use of feeding strikes is an accurate indicator of *Artemia* ingestion in 42-day-old juvenile seahorses under the experimental conditions described. There were no significant differences among the efficiency indices in the three-day observation ($F_{8,30} = 0.346$, $P = 0.940$) (Table 2.4-2).

7-day-old juveniles

There were no significant differences in feeding efficiency ($F_{4,10} = 0.461$, $P = 0.763$) or number of strikes ($F_{4,10} = 2.317$, $P = 0.128$) during the single-day observation, which supports the information from the first experiment on older fish (Table 2.4-2).

None of the efficiency indices were significantly different from 1.0 in any treatment on any day for both ages (Student's t-test, $P > 0.05$).

2.4.2 Effect of tank colour (white, yellow, red, blue and black) on growth, survival and *Artemia* ingestion by juvenile *H. abdominalis*

The results show that tank colour did not affect growth or survival over a 6-week period. There were no significant differences in initial length ($F_{5,12} = 1.43$, $P = 0.28$), final length ($F_{5,12} = 0.55$, $P = 0.73$), initial weight ($F_{5,12} = 0.78$, $P = 0.58$), and final weight ($F_{5,12} = 1.42$, $P = 0.28$) of the juvenile seahorses, among the treatments. On the final day of the experiment there were no differences in survival among the treatments ($F_{5,12} = 0.73$, $P = 0.61$) (Table 2.4-4).

Table 2.4-4 Initial and final length, initial and final wet weight, survival and specific growth rate (mean ± 1 S.E. of three replicates per treatment) of 3-day-old *H. abdominalis* juveniles exposed to one of six different tank colours in a 6-week growth trial. Each tank was fed *Artemia* 14 % BW d^{-1} (dry weight *Artemia*: wet weight fish).

Tank colour	Clear	White	Yellow	Red	Blue	Black
Initial length (mm)	17.0±0.1	17.0±0.0	17.0±0.1	17.0±0.1	17.0±0.1	17.0±0.10
Initial weight (mg)	8.7±0.3	8.9±0.2	9.3±0.0	8.9±0.3	9.1±0.4	9.3±0.2
Final length (mm)	38.0±0.9	39.0±0.6	38.0±0.6	38.0±1.6	36.0±0.9	38.0±0.7
Final weight (mg)	87.6±7.0	92.0±3.2	88.2±1.8	103.6±6.9	81.4±7.1	96.6±9.6
Survival (%)	66.6±7.8	73.3±3.9	86.7±6.7	75.8±4.6	80.0±13.3	71.1±8.9
SGR (% day^{-1})	5.5±0.2	5.6±0.1	5.4±0.1	5.8±0.2	5.2±0.2	5.6±0.2

Notes: The use of superscripts has been omitted as no significant differences were observed among treatments (one way ANOVA, $P > 0.05$).

Effect of tank colour (white, yellow, red, blue and black) on Artemia ingestion

There were no significant differences in *Artemia* ingestion among treatments, for week one ($F_{10,36} = 0.091$, $P = 1.000$), week three ($F_{10,36} = 1.557$, $P = 0.160$) or week five ($F_{10,36} = 0.601$, $P = 0.803$) during the three-day observations (Table 2.4-5).

Table 2.4-5 *Artemia* ingestion as feeding strikes (mean ± 1 S.E. of three replicates per treatment) in each tank colour for each of three sample days during week one, three and five of the 6-week growth trial. Feeding strikes were recorded from one randomly selected fish per tank per sample day.

Colour	Week one			Week three			Week five		
	Sample one	Sample two	Sample three	Sample one	Sample two	Sample three	Sample one	Sample two	Sample three
Clear	17.2±4.5	14.3±5.8	13.1±9.5	27.0±7.2	24.9±10.6	19.7±7.7	13.8±8.3	15.4±7.8	18.7±8.8
White	17.3±4.1	14.0±4.0	13.0±1.7	30.0±2.6	22.3±2.3	27.7±4.7	22.3±3.3	21.7±3.5	17.7±6.9
Yellow	22.3±1.2	15.3±1.5	18.3±1.5	42.3±3.7	20.0±2.3	25.3±9.9	31.0±1.5	19.0±0.6	18.0±2.5
Red	17.0±8.0	14.7±6.9	16.0±3.2	37.3±6.5	47.0±1.0	54.0±8.5	25.3±5.2	13.3±3.4	15.3±1.9
Blue	21.3±5.8	18.0±4.0	21.0±7.5	32.3±2.3	32.3±2.7	43.7±8.1	19.3±6.0	9.3±5.9	12.3±7.3
Black	15.3±3.7	7.0±3.0	9.3±6.6	41.0±3.8	46.3±2.4	39.7±6.9	12.3±5.0	12.7±4.8	15.7±3.4

Notes: Values in samples 1-3 were analysed together by an orthogonal ANOVA.
The use of superscripts has been omitted as no significant differences were observed among treatments ($P > 0.05$).

Colour conditioning

The results indicated a marked preference for the clear section rather than the colour treatments. This seahorse distribution in the cylinder did not change as shown by the lack of significant differences across the treatments in the 5-min observation *($\chi^2 = 2.51$, $df = 5$, $P = 0.77$) and the 10-min observation ($\chi^2 = 5.33$, $df = 5$, $P = 0.37$).*

2.5 Discussion

Across the eight colours tested fish were able to detect and ingest *Artemia* with no clear consistent differences found among any particular colour treatments. In the short-term trials the number of feeding strikes appeared to be influenced by tank colour on individual days although there was no consistent trend across sample days. As the use of feeding strikes appears to be an accurate measurement of *Artemia* ingestion in both ages tested, 7 and 42-day-old juveniles, the variability in the data may be due more to the varying response of individual fish to the change from holding tanks to a new coloured background than to the treatment colour itself. The variability among some of the replicates in the present study was quite high and this may be attributed to the change from one background colour to another and the impact that has on stress and ability of fish to adapt to the feeding under the new conditions in the short term. Moreover a larger sample size could contribute to the reduction of the variability; unfortunately the duration of activities involved in each observation day restricted the number of replicates. Other studies have indicated that early stage fish may take a number of days to condition to new culture conditions (Ostrowski, 1989). Hence the results in the short-term experiments may have only provided an instantaneous description of how fish initially responded to the new tank colour contributing to the observed variability among individuals. The 6-week trial would therefore appear to be a better indicator of how fish adapt and grow, and be more comparable to commercial holding.

The growth and survival of 3-day-old juvenile *H. abdominalis* was similar in all six tank colours tested over 6 weeks, indicating that over this period the fish did not appear to be adversely influenced by any of the tank colours tested. Such results suggest that the variable response in individual fish to exposure to a coloured background over a short term may be overcome quickly by acclimation to the new colour. The two-weekly measures of *Artemia* ingestion in colour treatments (Table 2.4-5) somewhat supports this suggestion as no significant differences in *Artemia* ingestion among colour treatments was found across three sample points (weeks one, three and five). On each sample day the amount of *Artemia* ingested was similar across colour treatments; while this may have been feeding rate limited it did not appear so as excess *Artemia* was always evident in all tanks at the end of the photoperiod.

In some respects these findings do not fully support the work of Woods (2000). While the author agrees that older fish (28-42-day-old) do not appear to be influenced by tank colour, in the present study 7-day-old seahorses displayed similar results compared to older seahorses, unlike the results of Woods (2000) who found that 7-day-old fish showed a greater strike rate in clear or white tanks compared to black tanks.

The present study demonstrated that under the experimental conditions used, 7-day-old and 42-day-old seahorses accurately ingested *Artemia* allowing feeding strikes to be used as a visual assessment of prey intake. The strike success in this study was higher than that of Woods (2000) and interestingly the *Artemia* size was smaller (570 µm c.f. 850 µm) but higher in density (100 *Artemia*/3-l c.f. 50 *Artemia*/2-l). Validation of the feeding strike technique for both age groups provided an accurate method of measuring *Artemia* ingestion in the long-term growth experiment.

There were differences in the magnitude of the data between the two studies. The strike rates and capture success in 28-42-day-old fish in the present study were 0.0 - 5.3 strikes min^{-1} (100 *Artemia*/3-l tank) and 90-116 % while in Woods (2000) they were c. 4-5 strikes min^{-1} (50 *Artemia*/2-l tank) and c. 70 %+ respectively. Also, the strike rates and capture success in 7-day-old fish in the present study were 0.2-3.4 strikes min^{-1} (100 *Artemia*/3-l tank) and 100-115 % while in Woods (2000) they were c. 2.5 - 4.5 strikes min^{-1} (50 *Artemia*/2-l tank) and 35-80 % respectively.

Light has been shown to be an important factor relative to feeding responses under different background colours (Browman and Marcotte, 1987). The intensity of 4.8 µE s^{-1} m^{-2} at the water surface used in this study is similar to the natural environment of this species and to the study of Woods (2000) and is within the range of light intensities that did not influence growth in juvenile *H. abdominalis* in a study by Ouyang (2005). Similarly studies on other seahorse species have also shown an ability to feed well under a wide range of light intensities (James and Heck, 1994; Wong and Benzie, 2003).

The interaction between tank colour and prey colour may also influence food intake in fish (Denderinos *et al.*, 1984). In this study *Artemia* were enriched with Super Selco® which would produce a light coloured gut in the *Artemia* in contrast to the microalgal enrichment resulting in a dark coloured gut in the study by Woods (2000) who suggested that this may enhance the contrast of *Artemia* in clear tanks. Such a difference may go some way to explain the differences in the findings of that study.

While some fish species perform better in light coloured (Papoutsoglou *et al.*, 2000; Tamazouzt *et al.*, 2000) or dark coloured (Ostrowski, 1989; Naas *et al.*, 1996; Martin-Robichaud, 1998) tanks, *H. abdominalis* appears to adapt equally well to a range of colours, especially over a prolonged period. Such adaptability provides some flexibility in husbandry approaches to select colours, which allows operators to more easily observe fish within the tanks or provide a background to promote skin colour changes in the fish (Wardley, 2001).

Short-term there was a reasonable amount of variation among sample days for any given colour making it difficult to identify any clear preferences in colour, unlike the study of Woods (2000) who showed that 7-day-old juveniles of this species displayed a higher prey strike rate in clear or white tanks compared with black tanks. Other fish species such as grouper *Epinephelus suillus* (Duray *et al.*, 1996), scaled carp *Ciprinus carpio* (Papoutsoglou *et al.*, 2000) and Eurasian perch *Perca fluviatilis* larvae (Tamazouzt *et al.*, 2000) also showed a preference for light over dark coloured tanks. In contrast mahi mahi *Coryphaena hippurus* (Ostrowski, 1989) and milkfish *Chanos chanos* (Duray, 1995) performed better in dark coloured tanks compared to light coloured tanks.

At the end of the 6-week trial a colour-conditioning test was used to determine if seahorses are conditioned to their tank colours during culture and display a spatial preference to this colour when offered a choice. Juvenile seahorses did not appear to show any colour conditioning after failing to show a preference for their culture colours in the multicolour wrapped Perspex cylinder as they tended to congregate in the clear section. While this may be interpreted as a positively phototactic response (as light could penetrate the clear section more readily) (Woods, 2000) all colours were exposed to the same overhead light intensity via a clear section along the top of the Perspex tube.

This study suggests that tank colour does not appear to affect the *Artemia* ingestion, growth or survival of early juvenile *H. abdominalis* seahorses under the experimental conditions described. Final results indicated that tank colour might not be critical in juvenile seahorse culture as they are with larval marine fish culture. However, some caution about these conclusions is needed because of the variability in the data. Further research could examine the interactions of tank colour and light intensity, retinal structure of the eye, stress response to environmental colour and colour preferences in seahorses.

2.6 Acknowledgements

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, Tasmania for providing the experimental fish and information. The author was financially supported by Consejo Nacional de Ciencia y Tecnologia, Mexico with the additional support of Universidad Marista de Merida, Mexico.

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CHAPTER 3

EFFECT OF WATER TEMPERATURE ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

3 EFFECT OF WATER TEMPERATURE ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

3.1 Abstract

In order to assess the feasibility to culture *Hippocampus abdominalis* in temperatures within the species's natural range (8-24 °C) and above it (26°C), the effect of four water temperatures (17 °C, 20 °C, 23 °C and 26 °C) on *Artemia* ingestion, growth and survival of early juvenile pot-bellied seahorse was investigated in two 6-week experiments. The objective of the first experiment was to examine the effect of water temperature (after an acclimation protocol) on seahorses fed with enriched *Artemia* at a fixed rate of 14 % initial body weight day^{-1} (dry weight *Artemia*: wet weight fish), while the objective of the second experiment was to review the previous experimental design by extending the acclimation protocol and adjusting the feeding rate according to daily mortality and weekly growth. At the end of first experiment, the highest survival was registered at 17 °C (mean ± 1 S.E.) at 73 ± 7 % compared to 20 °C and 23 °C, at 53 ± 3 % and 51 ± 6 % respectively. At 26 °C, 100 % mortality was reached on day 12. Seahorses cultured at 20 °C were longer and heavier than those at 17 °C, although not significantly different to 23 °C. At the end of the second experiment there were no differences in survival of juveniles cultured at 17 °C, 20 °C and 23 °C. At 26 °C 100 % mortality was reached on day 15. Juveniles grew better at the higher temperatures (20 °C and 23 °C) than at 17 °C. This study is the first to test a temperature of 26 °C on this species which showed not tolerance to this temperature. No effect on *Artemia* ingestion was found in either experiment. When food was not a limiting factor this species showed a much wider temperature range for growth in captivity than previously thought.

Keywords: *recirculation system; acclimation protocol; feeding activity; fish condition, temperature tolerance*

3.2 Introduction

Temperature is the most important single factor influencing fish growth (Tucker, 1998). Food consumption and food conversion efficiency can be improved by optimising culture temperature, which can lead to growth improvement in teleosts (Jonassen *et al.*, 2000) including the family Syngnathidae (Lin *et al.*, 2006; Silva *et al.*, 2006).

Temperature experimentation on juvenile fish can be focused on aspects such as temperature extremes (Tucker, 1998) and optima for species (Katersky and Carter, 2005). Regarding seahorse culture, successful rearing of *Hippocampus kuda* was achieved under laboratory conditions using a temperature range of 26.5-28.0 °C (Anil *et al.*, 1999), while Scarratt (1996) reported the use of 75 °F (23.8 °C) on *Hippocampus erectus* culture in captivity. Such difference in temperature ranges in these seahorses, considered warm-water species, indicates a species-specific temperature requirement in seahorse culture. *Hippocampus abdominalis* has been an experimental model in syngnathid research for the last 15 years. This species is a temperate water species that experiences a temperature range of 8–24 °C in the wild (Woods, 2001) while a temperature range of 10-19 °C has been used in captivity (Woods, 2000b).

A temperature range of 17-18 °C has been used for *H. abdominalis* experiments on general husbandry (Thomson, 1999; Adams *et al.*, 2001; Wardley, 2001; Florent, 2003; Shapawi and Purser, 2003; Woods, 2003a; Ouyang, 2005) and most nutritional studies (Woods, 2003b; Woods and Valentino, 2003; Woods, 2005; Wilson *et al.*, 2006). However, most of these studies have been conducted on late juveniles, with only a few experiments conducted under different temperature regimes (Leef, 2001; Woods, 2005) or on early stage juveniles (Woods, 2000a; Florent, 2003). The commercial scale seahorse culture at Seahorse World Pty. Ltd. in Tasmania also uses a temperature of 18 °C (Seahorse World Pty. Ltd. per. comm.). Woods (2001) conducted a study in which the effects of a temperature range (12, 15, 18 and 21 °C) on growth and survival was examined. The author found improved growth of this species at higher temperatures (18 and 21 °C) compared to lower temperatures (12 and 15 °C), while mortality was not affected by these temperatures.

The initial allocation of fish to temperatures used in experiments has been conducted using techniques such as direct transfer (McCarthy *et al.*, 1998; Sheng *et al.*, 2006; Silva *et al.*, 2006) or the gradual acclimation (Suneetha *et al.*, 1999), at asymmetric rates (Aune *et al.*, 1997; Jonassen *et al.*, 2000) or using equal transfer rates (Jonassen *et al.*, 1999; Katersky and Carter, 2005; Person-Le Ruyet *et al.*, 2006). Research regarding the effect of water temperature on seahorse culture has provided limited information on the temperature acclimation prior to the trials (Woods, 2001; Wong and Benzie, 2003).

Early stage fish research requires the use of specific techniques to measure the physiological response of animals to the factors tested. This specificity is related to the small size of the fish; it is not always possible to collect enough sample materials to conduct conventional techniques such as proximate analyses of protein and fat which are used to estimate the nutritional response of fish. Instead techniques such as the determination of the carbon and nitrogen index have been found to be an accurate indicator of the condition of fish. Protein has a carbon and nitrogen ratio close to 3; for instance in a tissue sample from fish in good condition it is expected to find protein and lipids with a carbon and nitrogen ratio greater than 3. In contrast, in poor-condition or starved fish the lipids are metabolized and the carbon and nitrogen ratio decreases (Westernhagen *et al.*, 1998). Similarly, low moisture content has also been associated with good condition in early stage fish as nutritionally stressed fish consumes body protein (which is replaced with water) in order to maintain homeostasis (Shackley *et al.*, 1993).

Woods (2001) reported a positive effect on growth of late juveniles of *H. abdominalis* cultured in temperatures up to 21 °C. However, no study has tested the effect of temperature on early juveniles of this species, despite the importance of temperature on early life-stages in marine fish culture (Yufera *et al.*, 1993). The primary aim of this chapter was to determine the effect of a selection of temperatures (17, 20, and 23 °C) within the natural range (8-24 °C) experienced by *H. abdominalis* and one temperature above that range (26 °C), on growth and survival in early juveniles. The specific objectives of this chapter are:

- To examine the effect of two different acclimation periods (24 h and 48 h prior to a transfer increase of 3 °C) on the growth and survival of *H. abdominalis* transferred

to a range of water temperatures.

- To examine the C: N ratio and moisture content as indicators of fish condition in response to water temperatures.

3.3 Materials and Methods

3.3.1 System design and general methods

Juvenile seahorses were transported in seawater and oxygen-filled plastic bags inside an insulated container from a commercial seahorse farm (Seahorse World Pty. Ltd. Beauty Point) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. Experimental fish were captive bred at a water temperature of 17 °C and a salinity of 32 ppt (g l^{-1}). After a 15 min temperature acclimation period the juveniles were allocated to a 20-l holding tank under the same conditions, before being distributed to 3-l tanks. Four separate 35-l recirculation systems were used to maintain four temperatures (Appendix one, diagram three). Each system comprised four 3-l tanks connected to a biofilter comprised of two stacked 22-l plastic containers. The upper container was filled with 40-mm bio balls and its floor area perforated every five centimetres to allow the outflow water from the tanks to trickle down to the container below. This lower container was used as a water reservoir in which was installed a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) that provided an inflow of approximately 2.5 l hr^{-1} $tank^{-1}$ of 20μm-filtered seawater. In reservoirs of three systems a heater was set to meet the desired temperatures. The remaining system met the desired temperature of 17 °C with the room air conditioning set at that temperature.

Both experiments were each conducted for 6 weeks and the seahorses utilised were combined from three different broods in each experiment. During the experiments the tanks were inspected daily for mortalities and any excess food and faeces were siphoned to waste. A 12:12 (L:D) photoperiod (lights on at 08:00 h, lights off 20:00 h) was provided by an overhead timer-controlled cool white fluorescent light 35 W (General Electric Company), providing a light intensity of 4.8 μE s^{-1} m^{-2} at the water surface. Continuous aeration was provided by flexible plastic tubing, ending with a 4-l h^{-1} plastic water-dripper (Neta®) acting

as an air stone. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish. For the determination of pH, TAN, nitrite and nitrate, a colorimetric saltwater liquid test kit (Aquarium Pharmaceuticals Inc.) was used. Salinity and temperature were monitored every 24 h while TAN, pH, nitrite and nitrate were recorded every 48 h during both experiments. Water quality was very similar in both experiments recording the following values: salinity (mean ± 1 S.E.) 32.7 ± 0.1 ppt, pH 7.8 (range 7.5-8.0), dissolved oxygen >75 %, TAN < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1}, nitrate < 5 mg l^{-1}. Salinity and temperature were monitored every 24 h and TAN, pH nitrite and nitrate were recorded every 48 h during both experiments. Initial and final length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a submerged plastic-covered 1-mm interval scaled sheet. Initial and final wet weight of seahorses (as well as the weekly bulk weights for the second experiment) was measured on an analytical balance and recorded to the nearest 0.0001g. Fish were purged for 24 h before each weighing.

3.3.2 Effect of temperature on growth and *Artemia* ingestion of *H. abdominalis*, over a 6-week period following a temperature-acclimation of 3 °C every 24 h

Two hundred and forty juvenile seahorses were randomly selected from 520 fish from three different broods (n = 180, n = 240, n = 100; 2, 1 and 0.5-day-old respectively) produced at Seahorse World Pty. Ltd. Four temperatures were maintained during the course of the experiment: 17 °C (treatment reference), 20 °C, 23 °C and 26 °C. Prior to the start of the experiments, seahorses were transferred from 17 °C, to the next temperature in 3 °C increments (after a 15 min temperature acclimation) every 24 h until juveniles were allocated to all the temperatures used in this experiment (24 h to 20 °C, 48 h to 23 °C, 72 h to 26 °C). Fifteen juveniles per tank were stocked into each of 16 3-l transparent tanks (four replicate tanks per treatment) and the length and wet weight of individual fish were recorded on day zero. Attachment substratum for the fish was provided by a weighted bundle of 55 nylon monofilament segments with a length of 139.21 mm ±1.51 mm (mean ± 1 S.E.). The fish were fed live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % initial body weight per day (BW d^{-1}) (dry weight *Artemia*: wet weight fish), which was divided into

two equal sized meals. Feeding occurred at 10:00 and 16:00 h. Screens (150 µm) were placed over the outlet of the tanks to prevent the loss of *Artemia* during the day. Feeding time was considered from 08:00 to 19:00 h daily, at the end of this period the screens were replaced with 500 µm screens to flush out the remaining unenriched *Artemia* overnight. *Artemia* flushed from tanks were collected in a central screen. The same amount of food was supplied in all the tanks throughout the experiment, and was not adjusted for mortalities or growth. After 6 weeks, the surviving seahorses were counted and their weight and length were measured. Mean specific growth rate (SGR) of seahorses in each tank was calculated by: SGR (% day^{-1}) = $[(\ln W_f - \ln W_i)/t] \times 100$, where W_f = final weight, W_i = initial wet weight, and t = number of days. Coefficient of variation (CV) of final fish body weight (BW) was calculated (Kestemont *et al.*, 2003) followed by size heterogeneity = CV_{wf}/CV_{wi} ; where $_{wf}$ = final weight, $_{wi}$ = initial wet weight, and CV = coefficient of variation (100 S.D./ mean).

A one-way analysis of variance (ANOVA, SPSS 11.5) was used to compare the means among treatments of survival, initial length, final length (mm), initial weight, final wet weight (mg), coefficient of variation (fish body weight g), size heterogeneity (fish body weight g), Fulton's K and SGR (% day^{-1}). A level of $P < 0.05$ was considered statistically significant. Levene's Test and residual plots were used to test homogeneity of variance. Tukey's HSD post hoc test was used to identify differences among treatment means (SPSS 11.5).

3.3.3 Effect of temperature on growth and *Artemia* ingestion of *H. abdominalis*, over a 6-week period following a temperature-acclimation of 3 °C every 48 h

While the first experiment used a 24 h acclimation to each temperature increment, in the second experiment a longer 48 h acclimation was tested. Two hundred and forty juveniles were randomly selected from 330 fish from three broods (n = 100, n = 60, n = 170; 7, 6, and 4-day-old respectively) produced at Seahorse World. Pty. Ltd. The same methods described in first experiment were used with the exception the acclimation was extended and the fish were kept for 48 h rather than 24 h at each temperature during acclimation before being transferred to the next temperature (48 h to 20 °C, 96 h to 23 °C, 144 h to 26 °C). The fish

were fed live *Artemia* under the same conditions previously described for the first experiment, except that the rate was maintained at 14 % (BW d^{-1}) (dry weight *Artemia*: wet weight fish) throughout the entire experiment, by adjusting the food on the basis of daily mortality (the rations corresponding to mortalities were not fed to the remainder of fish) and weekly growth recorded from bulk measures of wet weight per tank. After 6 weeks, the surviving seahorses were counted and their weight and length were measured individually.

A one-way ANOVA (SPSS 11.5) was used to compare the means among treatments of: survival, initial length, final length (mm), initial weight, final wet weight (mg), coefficient of variation (fish body weight g), size heterogeneity (fish body weight g), moisture (%), C: N ratio, Fulton's K (K) and SGR (% day^{-1}). A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. Final weight data was natural-log transformed to satisfy homogeneity of variance requirements. Tukey's HSD post hoc test was used to identify differences among treatment means (SPSS 11.5). An orthogonal ANOVA (SPSS 11.5) was used to compare the means of weekly weights among treatments over the 6 weeks trial.

3.3.3.1 Moisture, nitrogen and carbon content

The small size of the juveniles did not meet the minimum quantity of tissue required to conduct conventional proximate analyses. Instead, post-experiment analyses were conducted to quantify moisture, nitrogen and carbon content in the carcass in order to determine if seahorses cultured at temperatures above 17 °C metabolised food more efficiently than those cultured at 17 °C. At the end of the experiment, one seahorse per tank (randomly selected) was euthanized with an overdose of benzocaine (400 mg l^{-1}), blotted dry and weight and length recorded. Each whole seahorse was freeze-dried until constant weight was achieved. In addition, as low moisture content has been associated with a good condition early stage fish (Shackley *et al.*, 1993), those dried samples obtained were used for moisture content by determining the difference from wet weight. The seahorses were then individually ground with a mortar and pestle for analysis of nitrogen and carbon by oxidation/IR detection, using a CHNS auto-analyzer. Fulton's K has been used in seahorses (Wong and Benzie, 2003) and specifically in *H. abdominalis* (Woods, 2003a; b; Woods and Valentino, 2003) on a wet

weight basis as an indicator of seahorse condition. At the end of the experiment Fulton's K was calculated using the formula $K = (W/L^3) \times 100$ where W = wet weight (g) and L = length (cm).

3.3.4 Effect of temperature on *Artemia* ingestion

Direct visual observations of the number of feeding strikes as a measurement of *Artemia* ingestion were undertaken during each of the two experiments. *Artemia* ingestion was recorded by randomly selecting a single fish per tank and counting the feeding strikes during a 3-min period, one minute after the food was introduced into the tank. Observations were undertaken on one seahorse $tank^{-1}$ day^{-1} for three consecutive days during weeks one, three and five of both trials.

An orthogonal ANOVA (SPSS 11.5) was used to compare the means of feeding strikes among treatments over the three-day observation (as orthogonal factor) on each sample week. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. A square root transformation was applied to data of week one of the second experiment to satisfy homogeneity of variance requirements.

3.4 Results

3.4.1 Effect of temperature on growth and *Artemia* ingestion of *H. abdominalis*, over a 6-week period following a temperature-acclimation of 3 °C every 24 h

There were no significant differences in either juvenile length ($F_{3,12} = 0.86$, $P = 0.48$) or wet weight ($F_{3,12} = 3.47$, $P = 0.05$) among treatments at the start of the experiment. After 6 weeks there were significant differences in length ($F_{2,9} = 4.56$, $P = 0.04$), wet weight ($F_{2,9} = 5.65$, $P = 0.02$) and specific growth rate ($F_{2,9} = 5.96$, $P = 0.02$) (Table 3.4-1). The juveniles cultured at 20 °C were longer and heavier than the ones cultured at 17 °C, but were not significantly different from the juveniles cultured at 23 °C. There were no significant differences in Fulton's K ($F_{2,9} = 1.830$, $P = 0.215$), coefficient of variation ($F_{2,9} = 1.485$, $P = 0.277$) or size heterogeneity ($F_{2,9} = 3.791$, $P = 0.064$). Survival in the seahorses cultured at 17 °C was

significantly higher than those cultured at 20 °C, and 23 °C ($F_{2,9} = 4.75$, $P = 0.03$). The survival of seahorses cultured at 26 °C decreased to zero on day 12 (Figure 3.4-1).

Table 3.4-1 Survival, initial and final wet weight, initial and final length, coefficient of variation, size heterogeneity, Fulton's K and specific growth rate (mean ± 1 S.E. of four replicates per treatment) of early juvenile *H. abdominalis* cultured at four different temperatures in a 6-week growth trial following a temperature-acclimation of 3 °C every 24 h.

Temperature	17 °C	20 °C	23 °C	26 °C
Final observed survival (%)	73.3±7.2^{a}	53.3±2.7^{b}	51.7±5.7^{b}	0
Initial individual weight (mg)	11.5±5.7^{a}	9.4±0.9^{a}	9.5±0.7^{a}	8.9±0.7^{a}
Final individual weight (mg)	76.4±5.0^{a}	117.4±10.4^{b}	99.3±9.5^{ab}	*
Coefficient of variation (final body weight g)	27.1±4.2^{a}	39.5±2.4^{a}	36.5±2.4^{a}	*
Size heterogeneity index (body weight g)	1.3±0.11^{a}	2.36±0.32^{a}	2.71±0.60^{a}	*
Initial length (mm)	17.8±0.1^{a}	17.7±0.1^{a}	17.9±0.1^{a}	17.9±0.1^{a}
Final length (mm)	35.6±0.9^{a}	40.6±1.3^{b}	39.1±1.4^{ab}	*
Fulton's K	0.160 ± 0.001^{a}	0.17 0± 0.003^{a}	0.160 ± 0.004^{a}	*
SGR (% day $^{-1}$)	5.3±0.1^{a}	6.6±0.3^{b}	6.1±0.3^{ab}	*

Notes: Means with different superscripts within a row are significantly different (one-way ANOVA, $P < 0.05$).

* No data due to mortality.

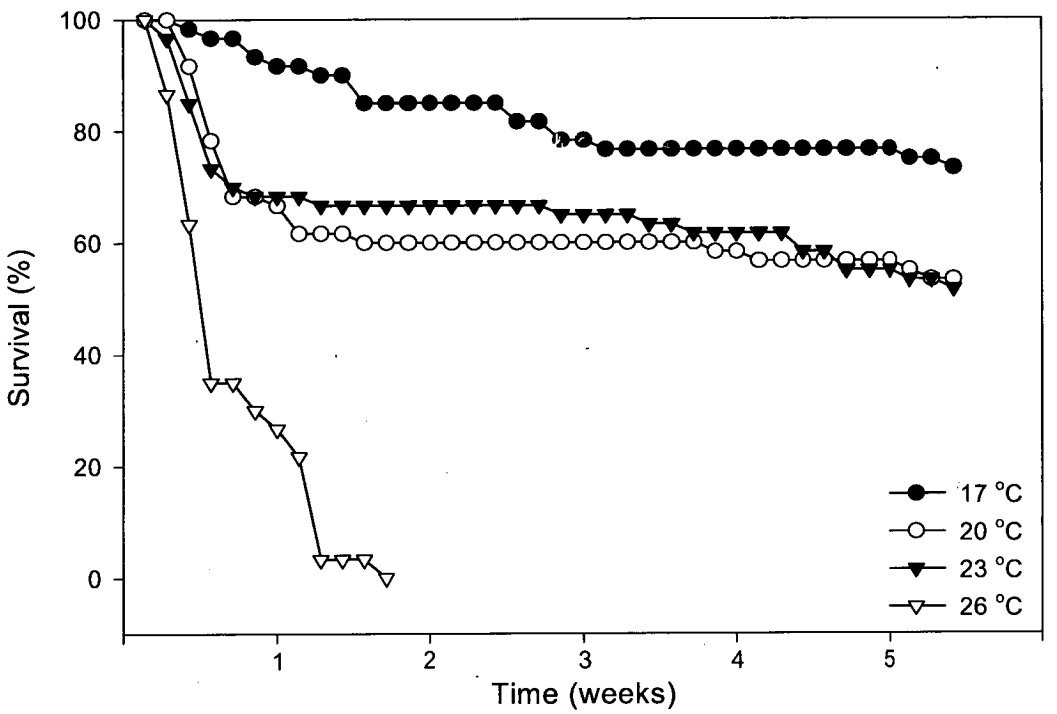

Figure 3.4-1 Daily survival (% mean of four replicates per treatment) of early juveniles of the seahorse *H. abdominalis* cultured at four different temperatures in a growth trial following a temperature-acclimation of 3 °C every 24 h. Juveniles were fed *Artemia* at a ration of 14 % BW d^{-1} with no adjustments throughout the experiment. Standard error bars were omitted to aid visualization.

3.4.2 Effect of temperature on growth and *Artemia* ingestion of *H. abdominalis*, over a 6-week period following a temperature-acclimation of 3 °C every 48 h

There were no significant differences in either juvenile length ($F_{3,12}$ = 1.146, P = 0.371) or wet weight ($F_{3,12}$ = 1.64, P = 0.23) among treatments at the start of each trial (Table 3.4-2). After 6 weeks, there were significant differences in length ($F_{2,9}$ = 17.170, P = 0.001), wet weight ($F_{2,9}$ = 9.780, P = 0.006) and specific growth rate ($F_{2,9}$ = 5.390, P = 0.029) (Table 3.4-2). The juveniles cultured at 23 °C and 20 °C were longer and heavier than the ones cultured at 17 °C. There were no significant differences in survival among the seahorses cultured at 17 °C, 20 °C or 23 °C ($F_{2,9}$ = 2.630, P = 0.126). The survival of seahorses cultured at 26 °C decreased to zero on day 15 (Figure 3.4-2). From the second bulk measuring to the end of the trial 20 °C and 23 °C fish were heavier than 17 °C (Fig 3). There were no differences in the coefficient of variation ($F_{2,9}$ = 0.746, P = 0.501) or the size heterogeneity ($F_{2,9}$ = 3.053, P = 0.097) among treatments at the end of the trial.

Table 3.4-2 Survival, initial and final wet weight, initial and final length, coefficient of variation, size heterogeneity, moisture, C: N ratio, Fulton's K, and specific growth rate (mean ± 1 S.E. of four replicates per treatment) of early juvenile *H. abdominalis* cultured at four different temperatures in a 6-week growth trial following a temperature-acclimation of 3 °C every 48 h.

Temperature	17 °C	20 °C	23 °C	26 °C
Final observed survival (%)	68.3±7.4[a]	68.3±8.3[a]	50.0±2[a]	0
Initial individual weight (mg)	15.1±0.8[a]	15.9±2.8[a]	13.9±0.4[a]	14.0±0.6[a]
Final individual weight (mg)	131.8±7.5[a]	199.9±6.1[b]	216.8±23.0[b]	*
Coefficient of variation (final body weight g)	40.3±4.1[a]	31.9±5.4[a]	31.2±7.4[a]	*
Size heterogeneity (body weight g)	1.60±0.17[a]	1.04±0.17[a]	1.00±0.25[a]	*
Initial length (mm)	19.9±0.5[a]	19.5±0.1[a]	19.3±0.2[a]	19.9±0.2[a]
Final length (mm)	40.5±0.8[a]	47.4±1.0[b]	48.3±1.3[b]	*
Moisture (%)	82.8±0.5[a]	81.9±0.2[ab]	80.4±0.4[b]	*
C:N ratio	3.70±0.1[a]	3.54±0.03[a]	3.78±0.14[a]	*
Fulton's K	0.170±0.005[a]	0.160±0.008[a]	0.160±0.006[a]	*
SGR (% day $^{-1}$)	5.1±0.2[a]	6.4±0.1[ab]	6.5±0.3[b]	*

Notes: Means with different superscripts within a row are significantly different (one-way ANOVA, $P < 0.05$).

* No data due to mortality.

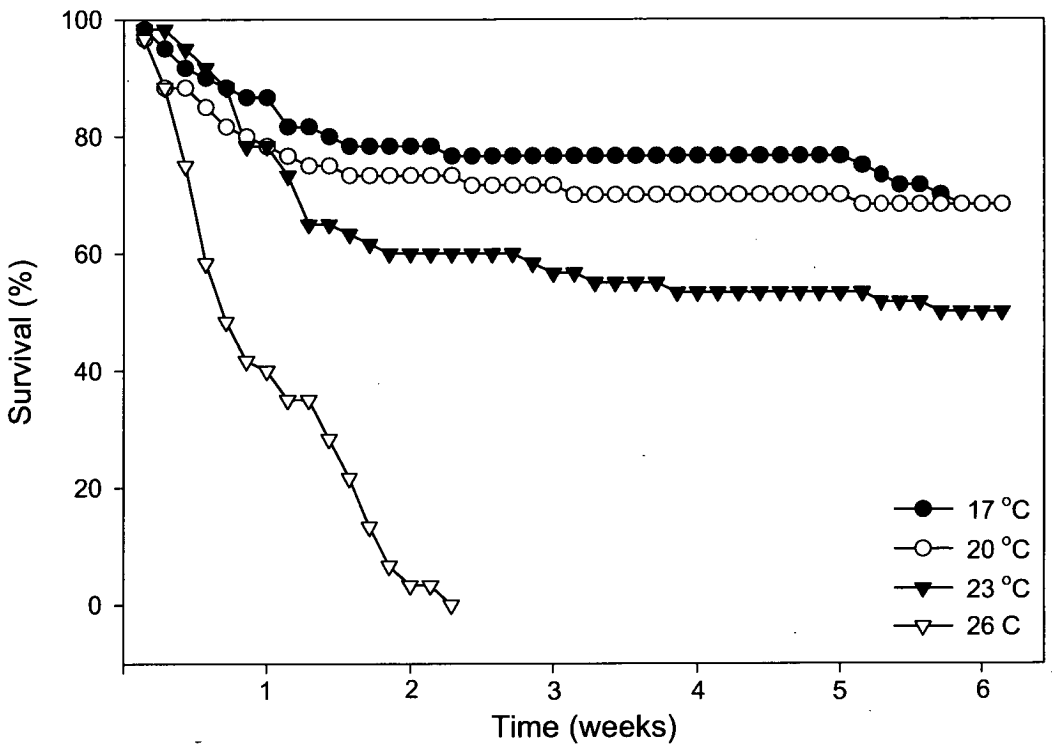

Figure 3.4-2 Daily survival (% mean of four replicates per treatment) of early juveniles of the seahorse *H. abdominalis* cultured at four different temperatures in a growth trial following a temperature-acclimation of 3 °C every 48 h. Seahorses were fed *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. Standard error bars were omitted to aid visualization.

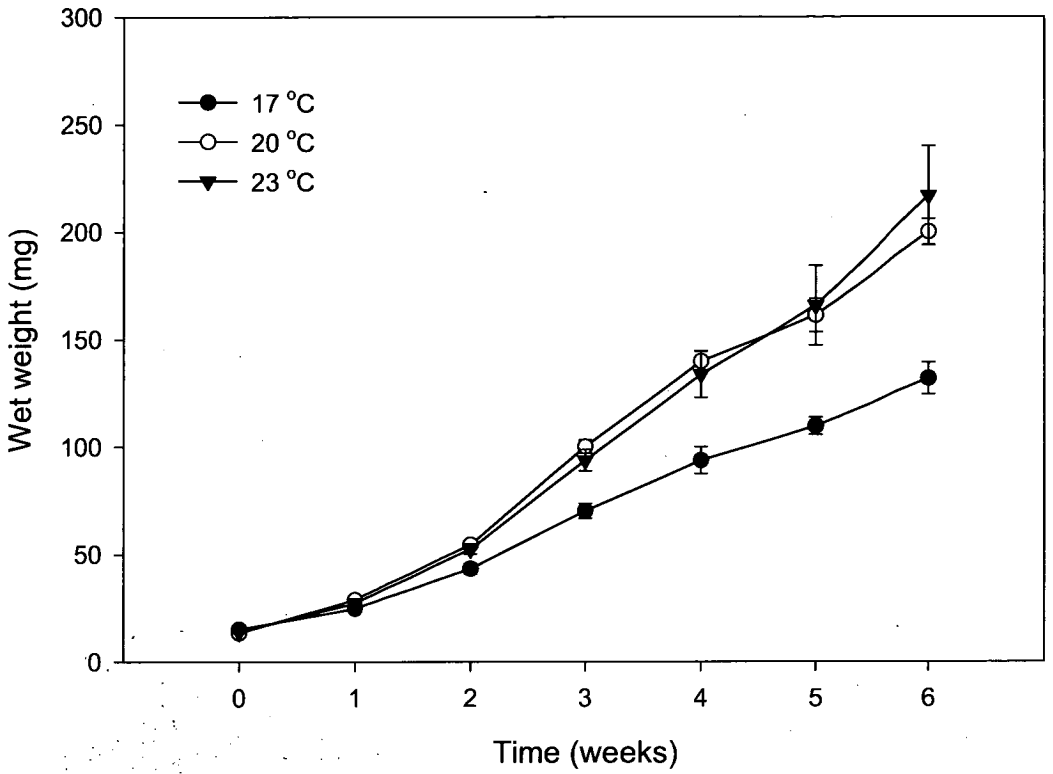

Figure 3.4-3 Wet weight of seahorses cultured at four different temperatures in a growth trial following a temperature-acclimation of 3 °C every 48 h. Seahorses were fed *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment ± 1 S.E.

3.4.3 Effect of temperature on *Artemia* ingestion

There were no significant differences in *Artemia* ingestion among treatments throughout the first experiment, during week one ($F_{4,27} = 2.610$, $P = 0.058$), week three ($F_{4,27} = 0.741$, $P = 0.573$) or week five ($F_{4,27} = 1.303$, $P = 0.294$) (Table 3.4-3). Similarly, in the second experiment there were no significant differences during week one ($F_{6,36} = 2.02$, $P = 0.08$), week three ($F_{4,27} = 1.029$, $P = 0.410$) or week five ($F_{4,27} = 0.547$, $P = 0.703$). It was not possible to record *Artemia* ingestion in juveniles cultured at 26 °C as all the seahorses died before the first *Artemia* ingestion during the first experimental sample was taken. In the first experiment during week one, seahorses cultured at 26 °C recorded the lowest overall mean of 6 strikes, which could have been a stress response before that treatment recorded 100 % mortality (48 h after the last observation) (Table 3.4-4).

Table 3.4-3 *Artemia* ingestion during the first experiment as strikes (mean ± 1 S.E. of four replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} in four different temperatures in a 6-week growth trial.

	Artemia ingestion								
Temperature	Week one			Week three			Week five		
	Day one	Day two	Day Three	Day one	Day two	Day three	Day one	Day two	Day three
17 °C	30.3±2.6	28.8±2.4	23.5±5.8	21.8±3.5	32.3±6.7	33.5±2.5	24.8±5.8	25.3±3.0	39.0±5.8
20 °C	34.0±3.1	40.3±2.8	27.8±5.1	40.0±5.1	33.5±7.6	32.5±4.2	29.8±7.0	23.5±6.8	17.3±1.7
23 °C	29.5±5.0	29.3±5.7	42.3±5.9	44.3±8.7	43.5±6.3	47.5±8.5	38.5±6.8	29.0±11.1	31.5±5.7

Note: The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P > 0.05$).

Table 3.4-4 *Artemia* ingestion during the second experiment as strikes (mean ± 1 S.E. of four replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} in four different temperatures in a 6-week growth trial.

	Artemia ingestion								
Temperature	Week one			Week three			Week five		
	Day one	Day two	Day Three	Day one	Day two	Day three	Day one	Day two	Day three
17 °C	9.5±1.3	11.8±1.8	13.0±1.7	15.3±5.6	9.8±4.1	10.8±4.8	16.8±3.1	12.8±4.6	19.0±1.6
20 °C	25.5±5.2	38.3±4.5	28.0±7.2	35.8±5.4	17.0±0.9	32.0±7.4	22.0±6.3	17.0±2.7	11.8±2.5
23 °C	28.3±9.0	55.0±6.7	32.0±6.3	23.0±3.9	20.0±3.6	20.8±4.3	27.0±8.0	22.5±6.3	23.3±2.8
26 °C	7.5±3.0	7.0±3.5	4.3±2.5	*	*	*	*	*	*

Notes: The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P > 0.05$).* No data due to 100 % mortality.

3.4.4 Moisture, nitrogen and carbon content

There were significant differences in moisture content of seahorses in different temperatures ($F_{2,9} = 7.90$, $P = 0.01$). The juveniles cultured at 23 °C contained less moisture than the ones cultured at 17 °C, the seahorses cultured at 20 °C contained a similar amount of moisture to those cultured at 23 °C and 17 °C (Table 3.4-2). There were no significant differences in C: N ($F_{2,9} = 1.56$, $P = 0.26$) or Fulton's K ($F_{2,9} = 0.94$, $P = 0.42$) among seahorse samples from the second experiment (Table 3.4-2).

3.5 Discussion

The present study is the first to report the response of *H. abdominalis* to temperatures of 23 °C and 26 °C in culture. In the first experiment the better growth produced at 20 °C compared to 17 °C could be explained by the interaction of feeding ration with temperature. The greater mean weight of seahorses at 23 °C and 20 °C combined with lower survival may suggest the selective mortality in some small and weak fish at elevated temperatures. The remainder of juveniles in tanks at 23 °C and 20 °C were a mixture of few small and weak fish with larger fish which possibly had better growth due to accessing more *Artemia* than juveniles at 17 °C in which more fish survived per tank. Although, at a non-significant level, this results were consistent with the higher coefficient of variation and the size heterogeneity index at 23 °C and 20 °C.

As the feeding ration of 14 % BW d^{-1} was not adjusted in the first experiment; there was a concern about the possibility of food limitation, despite excess *Artemia* in the tanks. However, this would appear unlikely as a daily feeding rate of 5 % of body weight has been used previously for *H. abdominalis* experimentation on newborns (Florent, 2003), juveniles (Wardley, 2001; Wilson *et al.*, 2006) and late juveniles (Woods, 2005). It has been found that the growth of fish cultured at higher temperatures can be negatively affected by limited food availability (Buckley *et al.*, 2004). Interestingly, the SGR of the seahorses in this experiment produced an increase from 17 °C to 20 °C, and then the SGR decreased at 23 °C. This suggests, despite the lack of significance, that a temperature of 20 °C could be more appropriate for juveniles than a temperature of 23 °C.

In the second experiment, the effect of temperature was separated from any possible limitation in food by feeding a constant rate of 14 % BW d^{-1} and adjusting the quantity on the basis of daily mortality and weekly growth. Despite this, a similar pattern of growth was produced in this experiment suggesting that initial concerns about ration were probably unfounded. However, in the second experiment fish at 23 °C showed significantly improved growth compared to juveniles at 17 °C.

In order to achieve a better understanding of the effect of temperature on early juvenile seahorse metabolism, whole-body samples were taken at the end of the trial. The poorer growth recorded in juveniles cultured at 17 °C was consistent with their significantly greater moisture content compared to seahorses at 23 °C which showed a better growth and a smaller amount of moisture. This relationship between better growth and low moisture content is an indicator of good condition in early stage fish (Shackley *et al.,* 1993). Although C: N ratios in samples of seahorses cultured at 23 °C appeared to be higher compared to the other treatments no significant differences were found. However, the overall C: N ratios were above 3.0, which indicate that the seahorses were not nutritionally stressed (Harris *et al.*, 1986).

During the first experiment, the higher survival at 17 °C could be due to juveniles being conditioned to this temperature before the experiment. While this appears somewhat unlikely because of the age of the fish, conditioning may have occurred during development in the male pouch. Literature on the temperature effect on seahorse culture has provided limited information regarding temperature acclimation prior to trials (Woods, 2001; Wong and Benzie, 2003). In the study conducted by Woods (2001) on late juvenile *H. abdominalis* there is no mention of an acclimation protocol. Although Wong and Benzie (2003) stated that juvenile *Hippocampus whitei* were acclimated over 48 h prior to each temperature used in that study, the authors did not provide a detailed protocol. In the present study, differences in survival among treatments presented similar trends in both experiments. However, among the remainder of treatments no differences in survival were observed in the second experiment as it was in the first experiment, possibly due to the extended acclimation period. Perhaps the 15 min acclimation used in this study was too abrupt as seahorses were transferred from 17 °C to 20, 23 and 26 °. However, mortality was present in the control

treatment (17 °C) which suggests that it was probably not the temperature changes which caused the mortality. In further research the use of an automatic temperature control could diminish temperature related stress by gradually transferring the fish from the reference temperature to the next temperature preventing the effect of the 3 °C change in 15 min.

The seahorses cultured at 26 °C died at approximately the same stage in both experiments despite an extended acclimation period, which indicated that 26 °C was outside the tolerance range at least after the acclimation periods used in the present study. In general, exposure of teleosts to an extreme temperature can alter the function of the cardiovascular system, nerves, proteins, and enzymes especially in juveniles of sensitive species (Tucker, 1998). Woods (2001) reported the presence of *H. abdominalis* in temperatures as high as 24 °C in the wild which is consistent with the findings of the present study in which *H. abdominalis* appeared to have an upper thermal tolerance for survival at some point between 23 °C and 26 °C. Therefore further research is needed to determine this temperature.

In the temperature study conducted by Sheng *et al.* (2006) the authors found that *Hippocampus trimaculatus* experiences two critical points for mortality. The first critical point at approximately day three after birth (first feeding) and the second critical point at approximately day 10 after birth when seahorses change their prey preference from copepod nauplii to adult copepods. It was suggested that the change affected *Artemia* ingestion and consequently survival. Comparisons to other seahorse studies are difficult to establish as the response of each species to environmental factors such as temperature or salinity can be age- and species- specific (Hilomen-Garcia *et al.*, 2003). In the present study *H. abdominalis* displayed only one critical point for survival in the initial 7-10 days after the temperature acclimation. This critical point was unlikely to be caused by first feeding or the change prey size as the rather larger size of the newborn *H. abdominalis* compared to the smaller *H. trimaculatus* newborns, allows them to prey directly on *Artemia* nauplii on the day of release from the male's pouch. While a proportion of this mortality may be due to handling and transfer stress (as some mortality was seen in the 17 °C reference temperature) the remainder may be due to the initial change in temperature as survival levelled after the initial 7-10 days mortality. In addition, the highest variability on survival across treatments was recorded during the first two weeks of the experiments.

The findings of the present study are consistent with Woods (2001), who found improved growth in late juveniles (80 mm, 0.7 g) of *H. abdominalis* with increasing temperature, although the highest temperature used in that study was 21 °C, and a maximum tolerance temperature was not determined. In contrast to the findings of the present study, Wong and Benzie (2003) reported an increase in length and wet weight of the temperate-water seahorse *H. whitei* when cultured at 26 °C, which can be explained by the geographical distribution of that species especially in the coastal area (seagrass beds in Cliffton Gardens, Sydney) where the fish were collected for that study. *H. whitei* covers a more tropical distribution (North-east coast of Australia, Solomon Islands) than the pot-bellied seahorse, which tends to populate the cooler waters of the southern coast of Australia and New Zealand. Therefore, it is not difficult to understand its better adaptation to high temperatures such as 26 °C compared to the poorer adaptation of *H. abdominalis* to 26 °C.

The present study considered a temperature of 17 °C to provide a close reference to the temperature of 18 °C used in *H. abdominalis* commercial scaled culture (Martinez-Cardenas and Seahorse World Pty. Ltd. pers. obs.) and a high temperature of 26 °C to assess its suitability for *H. abdominalis* culture. Based on the results it can be concluded that under the experimental conditions described in this study, *H. abdominalis* cannot be considered for the warm-water aquarium trade. However, it would be advantageous to examine the viability of the large scale rearing of pot-bellied seahorses at 23 °C after longer temperature acclimation protocols, which could optimise commercial culture, through improved growth rates.

Prolonged culture over subsequent generations at elevated temperatures such as 23 °C could potentially lead to temperature tolerance through selective breeding. However, the effect of elevated temperature on courtship, mating and pouch incubation is unknown. In addition, this study was based on constant temperatures as found in intensive recirculation systems in aquaculture. Therefore, there is a need in future studies to test the effect of temperature fluctuations relevant to flow-through systems or cage systems on early juvenile seahorse rearing.

3.6 Acknowledgements

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish. Special thanks to Ms Renee Florent, for her kind assistance on the improvement of earlier manuscript drafts and Mr Richard Scott Wallace for the valuable information provided on commercial scaled seahorse culture. The author was financially supported by Consejo Nacional de Ciencia y Tecnologia Mexico, with the additional support of Universidad Marista de Merida, Mexico.

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CHAPTER 4

EFFECT OF STOCKING-DENSITY ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

4 EFFECT OF STOCKING DENSITY ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

4.1 Abstract

The commercial culture of *H. abdominalis* experiences an increase in reproduction rates during the summer season systems resulting in higher than normal stocking densities in some facilities or a reluctance to attempt higher than normal densities in other facilities. This production peak can overwhelm the holding capacity of rearing systems. The effect of stocking-density on the response in growth, survival and *Artemia* ingestion of early juvenile *Hippocampus abdominalis* was examined in two experiments. In the first experiment, stocking-densities of 45, 30, 15 and 5 juveniles 3 l^{-1} were tested on newborns, (mean ± 1 S.E., 17.00 ± 0.07 mm in length and mean ± 1 S.E., 8.41 ± 0.19 mg in wet weight) over six weeks. Growth (length, weight, size heterogeneity and SGR) was independent of stocking-density. There were no significant differences in survival or *Artemia* ingestion among treatments at the end of the trial. Mortality displayed a peak in the first week of the trial. The second experiment aimed to remove the effect of early mortality as experienced in newborns during the first experiment, by using older juveniles (mean ± 1 S.E., 23.0 ± 0.3 mm in length and 25.0 ± 0.1 mg in wet weight) over a 4-week period. Three stocking-densities were tested (25, 15 and 5 juveniles 3 l^{-1}). There was no difference in growth. However, a mortality pattern similar to that in the first experiment was recorded. The results of this study suggest that early juvenile *H. abdominalis* can be cultured at higher stocking-densities than previously reported in literature, without compromising growth.

Keywords: *recirculation system; size heterogeneity; feeding activity; fish condition*

4.2 Introduction

The use of high stocking-densities in marine fish culture is required for cost-efficient commercial production of juvenile fish (Daniels *et al.,* 1996). However, the actual effect that stocking density has on fish is species-specific and can be negative, positive or neutral (Woods, 2003b). Seahorse density in their natural environment tends to be low with *H. abdominalis* among the species with the lowest mean densities recorded (0.007 animals per m^{-2}; Foster and Vincent, 2004). Therefore, under culture conditions an increase in stocking density would suggest an effect on pairing behaviour and feeding activities. However, commercial scale *H. abdominalis* culture is successfully conducted at a maximum stocking density of 100 adults per 1000-l tank and juveniles have been reared at stocking densities of 28, 000 fish in 2 m^3 Rathburn tanks (Seahorse World Pty. Ltd. pers. comm.). There is little information on seahorse stocking density and the few studies published have focused on early stages due to the occurrence of high mortalities. A major constraint in the development of marine fish culture has been the successful rearing of early stage fish (Daniels *et al.*, 1996; Odile *et al.*, 1996). Early-stage mortalities appear as an interaction among various factors such as early fish quality, chronic starvation (Shackley *et al.*, 1993) and negative social interaction (Hatziathanasiou *et al.*, 2002).

There are reports on the positive use of high stocking-density in marine fish larvae culture. Tagawa *et al.* (2004) found biogenical material in the culture media derived from the Japanese flounder (*Paralicthys olivaceus*) larvae beneficial for survival when fish were cultured at high densities, but not at low densities. The intensive culture of marine fish larvae also presents problems, such as poor water quality, diseases and abrasion. The deterioration of the culture environment can occur at a system level due to overloading of the biofilter (Alvarez-Gonzalez *et al.*, 2001). Poor in-tank water conditions may occur from inadequate maintenance of tanks (in some cases due to design misconceptions) and inadequate water exchange needed to remove metabolites. This lack of adequate water exchange usually generates zones with suboptimal concentrations of oxygen that can lead to high mortalities (Tucker, 1998). Other causes of mortality of early stages of cultured fish can be their high susceptibility to crowding stress (Van der Salm *et al.*, 2004) and the development of negative social hierarchies/cannibalism at high densities (Hatziathanasiou *et al.*, 2002). The culture of

early stage seahorses at high stocking-density has an advantage regarding negative social interactions, compared to other teleosts, as seahorses do not display cannibalistic tendencies (Bergert and Wainwright, 1997) or hierarchies (Woods, 2003b).

During experimentation with *H. abdominalis* a range of stocking densities has been used. Wilson (2006) utilized 1 late-stage juvenile seahorse l^{-1} (wet weight 1.17 g), Florent (2003) used 1 newborn seahorse l^{-1}, and Woods (2003b) considered 0.5 and 1 seahorse l^{-1} as suitable stocking-densities of seahorses for a variety of different sizes and ages (Woods, 2000a; 2003b; c; Woods and Valentino, 2003). Also Woods (2003b) conducted an experiment in which a range of stocking-densities (1, 2, and 5 seahorses l^{-1}) were tested. A reduction in survival and a greater incidence of physical interference (i.e. tail grasping and wrestling) was found among 5-month-old juveniles (72 mm in standard length and 0.5 g in wet weight) cultured at a stocking density of 5 seahorses l^{-1} compared to those at 1 and 2 seahorses l^{-1}. In other seahorse species such as *Hippocampus erectus*, stocking-density has been tested at 6 newborns l^{-1}, lowered to 1 seahorse l^{-1} after juveniles reached 35-day-old (Correa *et al.*, 1989). Wong and Benzie (2003) reported a lack of significant differences in growth of 3-month-old *Hippocampus whitei*, when testing a range of 0.5 to 1 seahorse l^{-1}. Most of those studies have reported low mortality rates as they have been conducted on late juveniles. This is not surprising as survival in late stage seahorses is more stable than in early stages. Also the mentioned studies have tested only a limited range of stocking densities. Therefore, one of the objectives of this study is to examine a greater range of stocking densities than reported in the literature on early juvenile *H. abdominalis*.

H. abdominalis does not present a conventional larval-stage as in many other marine fish species due to their specialized parental care, where the embryos develop inside the male's pouch. After approximately 28 days at 17 °C juveniles are released at birth as autonomous fish at which stage they are referred to as "newborns" in this study. Newborn seahorses are well-developed and able to feed on the day of release. Newborn seahorses are distinctive due to their rather large size (approx. length 17 mm in *H. abdominalis*) and relative smaller broods compared to other marine teleosts (Foster and Vincent, 2004), such as summer flounder *Paralichthys dentatus* (newborn length aprox. 3.5 mm) which are experimentally stocked up to 60 fish l^{-1} (King *et al.*, 2000), and Atlantic cod *Gadus morhua*, (length aprox. 5

mm) which has been reported to be cultured at stocking-densities as high as 300 fish l^{-1} (Baskerville-Bridges and Kling, 2000). Juvenile production is the most important factor required to establish the culture of a species with good aquaculture potential (Alvarez-Gonzalez *et al.*, 2001). *H. abdominalis* has been a useful seahorse model for aquaculture and biological research; it breeds small broods year round (Lourie *et al.*, 2004) which commercial scale facilities maintain at a density of approximately 400 newborns per 50-l tank (Seahorse World Pty. Ltd. pers. comm.). However, breeding peaks occur in summer and during particularly high productive seasons the water temperature is lowered in order to suppress mating (Seahorse World Pty. Ltd. pers. comm.) due to the lack of tank resources to accommodate broods. Seahorses spend a large proportion of their life attached to a diversity of materials (Foster and Vincent, 2004) particularly during the dark phase (Ouyang, 2005) or when not foraging for food (Karina *et al.*, 2006). Therefore, the availability of space during these periods depends on the availability of the attachment substrate in addition to free tank space used during swimming and foraging. The primary aim of this study was to examine the effect of higher stocking-densities than reported in the literature (or used commercially) on early juvenile survival and growth. The objective of this research was to compare the effect of different stocking densities on growth, *Artemia* ingestion and survival of early juvenile seahorses.

4.3 Materials and Methods

4.3.1 System design and general methods

Juvenile seahorses were transported in seawater and oxygen-filled plastic bags inside an insulated container from a commercial seahorse farm (Seahorse World Pty. Ltd., Beauty Point) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. Experimental fish were captive bred at a temperature of 17 °C in 32 ppt (g l^{-1}) seawater. On arrival the juveniles were allocated to a 20-l holding tank at the same conditions of birth. Transparent 3-l tanks arranged in a recirculation 100-l system were utilised for the experiments. The recirculation system included a biofilter comprised of two stacked 40-l plastic containers. The upper container was filled with 40-mm bio balls and its floor area perforated every five centimetres to allow the outflow water from the tanks to

trickle down to the container below. This lower container was used as a water reservoir in which was installed a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) that provided an inflow of approximately 2.5 l hr^{-1} $tank^{-1}$of 20µm filtered seawater. Continuous aeration was provided by flexible plastic tubing ending in a 4-l hr^{-1} plastic water-dripper (Neta®) acting as an air stone. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish. A 12:12 (L:D) photoperiod (lights on at 08:00 h, lights off 20:00 h) was provided by a timer-controlled 35 W overhead cool white fluorescent light (General Electric Company) producing an intensity of 4.8 µE s^{-1} m^{-2} at the water surface. Water quality was maintained for both experiments as follows (mean and range): water temperature 16.6 °C (16.0-17.5 °C), pH 8 (7.8-8.2), salinity 34.2 ppt (33-35 ppt) dissolved oxygen >75 %, total ammonia nitrogen (TAN) < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1} and nitrate < 5 mg l^{-1}. For the determination of pH, TAN, nitrite and nitrate, a colorimetric saltwater liquid test kit (Aquarium Pharmaceuticals Inc.) was used. Salinity and temperature were monitored every 24 h while TAN, pH, nitrite and nitrate were recorded every 48 h during both experiments. Initial and final length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a submerged plastic-covered 1 mm scaled sheet. Seahorses were gently dabbed on a paper towel to remove excess water prior to weighing; initial and final wet-weight of juveniles, and weekly bulk weight were measured on an analytical balance and recorded to the nearest 0.0001g. Fish were purged for 24 h before each weighing. The experiments were conducted for 42 and 31 days respectively, during which time the tanks were inspected daily for mortalities and any excess food and faeces were siphoned to waste. Water exchanges were conducted to replace siphoned water.

4.3.2 Effect of four stocking densities (45, 30, 15 and 5 fish 3 l^{-1}) on growth, survival and *Artemia* ingestion by early juvenile seahorses in a 6-week trial

From 385 juvenile seahorses collected from four different broods (n = 25, n = 60, n = 200 and n = 100; 4, 3, 2 and 1-day-old respectively), 320 were used in this experiment. Four densities: 45, 30, 15 and 5 seahorses 3 l^{-1} (15, 10, 5 and 1.6 seahorses l^{-1}), each with four replicates were arranged randomly for the experiment. After one day in the 20-l holding tank

at a density of approximately 20 juveniles l^{-1}, the appropriate number of juveniles for each 3-l tank were randomly selected, then distributed to each of 16 3-l transparent tanks (four replicate tanks per each treatment), after the length and wet weight of individual fish were recorded (day zero). Attachment substratum for the fish in all treatments was provided by a weighted bundle of 55 nylon monofilament segments with a length of 139.21 mm ±1.51 mm (mean ± 1 S.E.). The fish were fed live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % body weight day^{-1} (dry weight *Artemia*: wet weight fish) that was divided into two equal sized meals delivered at 10:00 and 16:00 h. Screens (150 µm) were placed over the outlet of the tanks to prevent the loss of *Artemia* during the day. Feeding time was considered from 08:00 to 19:00 daily, at the end of this period the screens were replaced with 500 µm screens to flush out the remaining unenriched *Artemia* overnight. The feeding rate was maintained throughout the entire experiment, by adjusting the food on the basis of daily mortality (the rations corresponding to mortalities were not fed to the remainder of fish) and weekly growth recorded from bulk measures of wet weight per tank. Mortalities were removed and recorded daily; these mortalities were not replaced as early juvenile seahorses are difficult to mark or tag.

After 6 weeks, the surviving seahorses were counted and their weight and length were measured individually. Mean specific growth rate (SGR) of seahorses in each tank was calculated by: SGR % day^{-1} = $[(\ln W_f - \ln W_i)/t] \times 100$, where W_f = final weight, W_i = initial wet weight, and t = number of days. Coefficient of variation (CV) of final fish body weight (BW) was calculated (Kestemont *et al.*, 2003) followed by size heterogeneity = CV_{wf}/CV_{wi} ; where $_{wf}$ = final weight, $_{wi}$ = initial wet weight, and CV = coefficient of variation (100 S.D./ mean).

A one-way analysis of variance (ANOVA, SPSS 11.5) was used to compare the means among treatments of survival, initial length, final length (mm), initial weight, final wet weight (mg), coefficient of variation (fish body weight g), size heterogeneity (fish body weight g), Fulton's K and SGR (% day^{-1}). A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. A square root transformation of size heterogeneity data was conducted to satisfy homogeneity of variance requirements. Tukey's HSD post hoc test was used to identify differences among treatment

means (SPSS 11.5).

An orthogonal ANOVA (SPSS 11.5) was used to compare weekly (time as orthogonal factor) mean wet-weights among treatments throughout the experiment. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. A natural-logarithm transformation was conducted on data of bulk wet-weights in both experiments in order to satisfy homogeneity of variance requirements.

4.3.2.1 Effect of four stocking densities on Artemia ingestion

Artemia ingestion was recorded by selecting a single fish per tank at random and counting the feeding strikes produced during a 3-min period, one minute after the food was introduced into the tank. Counting was undertaken on one seahorse/tank/day for three consecutive days in each of weeks two and four of the experiment.

An orthogonal ANOVA (SPSS 11.5) was used to compare the means of feeding strikes among treatments over the three-day observation (as orthogonal factor) on weeks two and four. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. A natural-logarithm transformation of data was conducted on week one in order to satisfy homogeneity of variance requirements.

4.3.3 Effect of three stocking densities (25, 15 and 5 fish 3 l^{-1}) on growth and survival of 21-day-old juvenile seahorses in a four-week trial

Four different broods and a total of 560 newborn seahorses (n = 139, n = 16, n = 246 and n = 159), were held for three weeks in a 20-l tank at a density of approximately 20 juveniles l^{-1}. Then 180 juveniles were randomly selected for this experiment. The three stocking densities of 25, 15, and 5 seahorses 3 l^{-1} (8.3, 5 and 1.6 seahorses l^{-1}), each with four replicates were arranged randomly for the experiment. The appropriate number of juveniles for each tank were selected, and then distributed to each of 16 3-l transparent tanks, after the length and wet weights of individual fish were recorded (day zero). The feeding protocol as described in

section 4.3.2. was followed . After four weeks, individual weight and length of the surviving seahorses were measured and statistical analyses were conducted as described in the first experiment. A natural-logarithm transformation of weekly bulk weights data was conducted, and a square root transformation of data of size heterogeneity was conducted, both in order to satisfy homogeneity of variance requirements.

4.4 Results

4.4.1 Effect of four stocking densities (45, 30, 15 and 5 seahorses 3 l^{-1}) on growth, survival and *Artemia* ingestion by early juvenile seahorses in a six-week trial

The results show that the stocking densities tested did not affect growth or survival over a 6-week period. There were no significant differences in initial length ($F_{3,12} = 2.82$, $P = 0.08$), final length ($F_{3,12} = 0.65$, $P = 0.59$), initial weight ($F_{3,12} = 2.47$, $P = 0.11$), and final weight ($F_{3,12} = 2.11$, $P = 0.15$) of the juvenile seahorses among treatments (Table 4.4-1). On the final day of the experiment there were no differences in survival (Figure 4.4-1) ($F_{3,12} = 0.14$, $P = 0.93$), specific growth rate ($F_{3,12} = 2.69$, $P = 0.09$) or size heterogeneity ($F_{3,12} = 1.368$, $P = 0.300$) among the treatments (Table 4.4-1). Also, there were no significant differences in any of the weekly bulk weights ($F_{18,84} = 0.284$, $P = 0.998$). However, the growth of fish at 45, 30 and 15 seahorse 3 l^{-1} reached a plateau after week five, while seahorses at 5 fish 3 l^{-1} maintained linear growth until the end of the experiment (Figure 4.4-2).

Table 4.4-1 Survival, initial and final length, initial and final wet weight, size heterogeneity and specific growth rate (mean ± 1 S.E. of four replicates per treatment) of early juvenile *H. abdominalis* cultured at four different stocking-densities in a 6-week growth trial.

Seahorses 3 l^{-1}	45	30	15	5
Final observed survival (%)	59.4±10.1	55.8±8.0	63.3±5.8	60.0±8.2
Initial individual weight (mg)	8.9±0.2	8.4±0.2	8.1±0.0	8.3±0.4
Final individual weight (mg)	71.0±1.5	75.6±5.1	64.4±3.6	92.7±15.4
Initial length (mm)	17.00±0.05	17.00±0.13	17.00±0.04	17.00±0.10
Final length (mm)	35.0±0.4	35.0±0.4	33.0±1.1	35.0±1.6
Size heterogeneity (body weight g)	1.4±0.1	1.4±0.2	1.9±0.3	1.6±1.0
SGR (% day^{-1})	4.94±0.03	5.22±0.21	4.93±0.13	5.67±0.34

Note: The use of superscripts has been omitted as there were no significant differences among treatments (one-way ANOVA, $P > 0.05$).

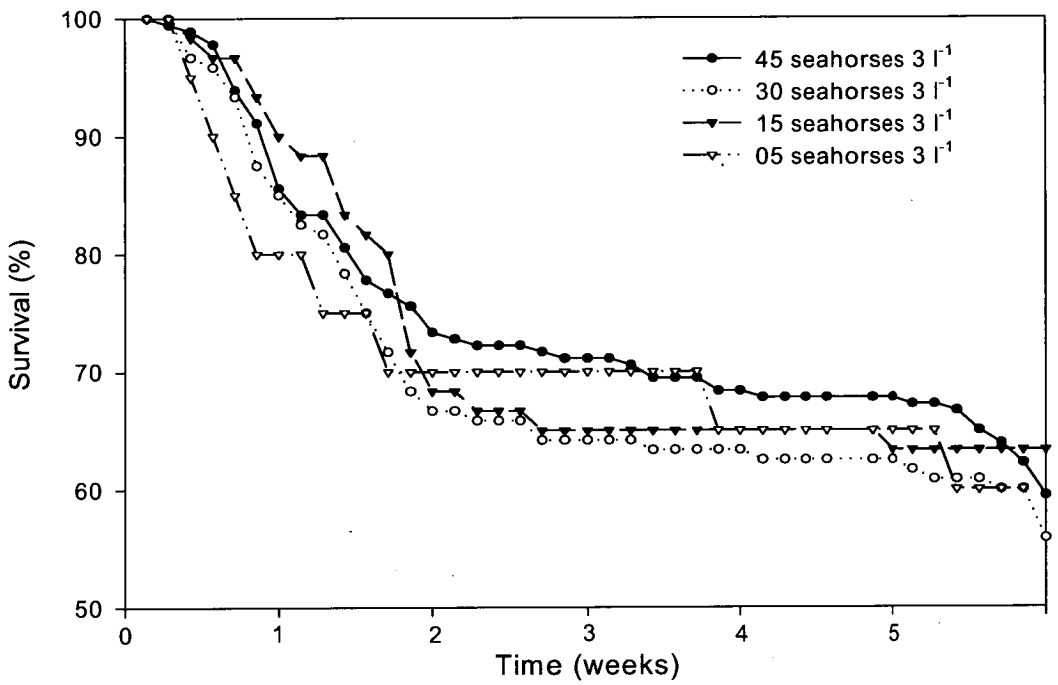

Figure 4.4-1 Survival of early juvenile *H. abdominalis* cultured at four different stocking densities. Seahorses were fed *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

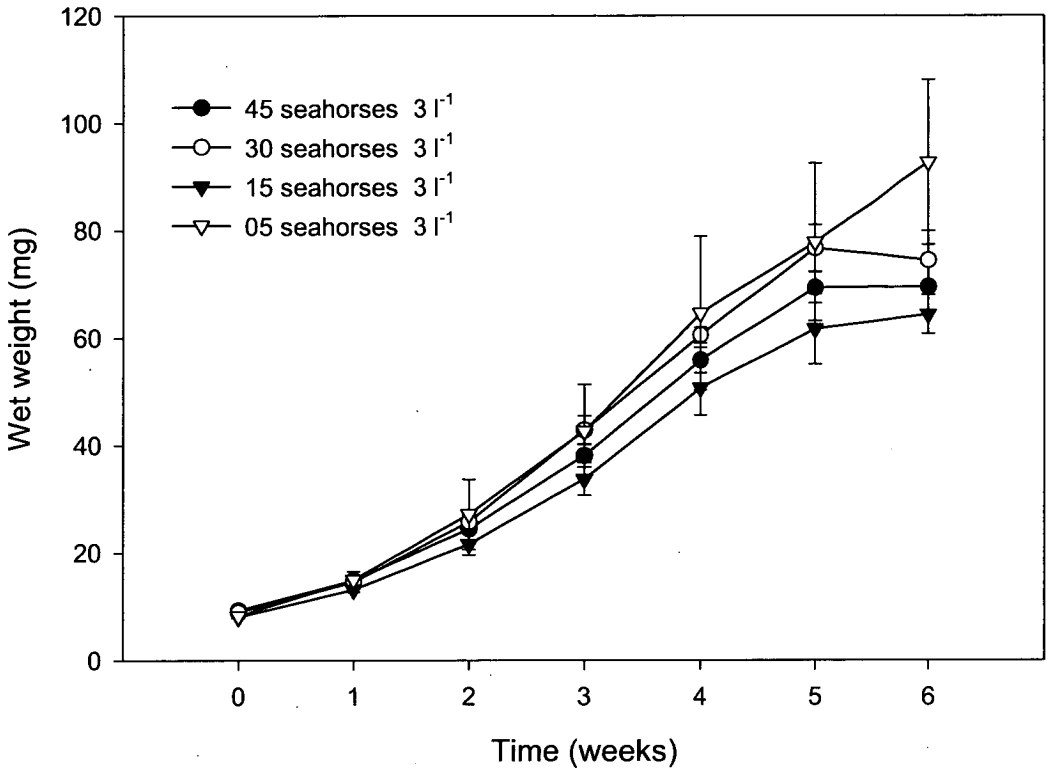

Figure 4.4-2 Wet weight of early juvenile seahorses *H. abdominalis* cultured at four different stocking-densities. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment ± 1 S.E.

4.4.1.1 Effect of four stocking densities on Artemia ingestion

Artemia ingestion was independent of stocking-density as there were no significant differences throughout the experiment during week two ($F_{6,36} = 0.14$, $P = 0.99$), or week four ($F_{6,36} = 0.51$, $P = 0.79$) (Table 4.4-2). On each sample day the mean *Artemia* ingested per fish was similar among densities.

Table 4.4-2 *Artemia* ingestion as strikes (mean ± 1 S.E. of four replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} at four different densities observed over three consecutive days in each of week two and four of the 6-week growth trial.

	Artemia ingestion					
Seahorses 3 l^{-1}	Week two			Week four		
	Day one	Day two	Day three	Day one	Day two	Day three
45	6.8±1.7	19.0±3.2	16.0±2.3	13.8±1.1	13.0±1.2	12.3±4.3
30	5.0±1.3	17.3±6.8	16.0±6.0	15.5±8.1	11.5±0.9	11.5±1.6
15	4.8±1.4	19.3±2.1	17.8±3.0	19.5±3.6	25.3±1.7	16.5±6.3
5	8.0±1.2	27.5±7.4	20.8±2.9	14.5±2.3	14.5±3.2	7.0±2.7

Note: The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P > 0.05$).

4.4.2 Effect of three stocking densities (25, 15 and 5 seahorses 3 l^{-1}) on growth and survival of 21-day-old juvenile seahorses

Stocking density did not affect growth or survival over the 4-week period. There were no significant differences in initial length ($F_{2,9} = 0.836$, $P = 0.465$), final length ($F_{2,9} = 3.06$, $P = 0.09$), initial weight ($F_{2,9} = 1.14$, $P = 0.36$), and final weight ($F_{2,9} = 0.96$, $P = 0.41$) of the juvenile seahorses, among treatments (Table 4.4-3). On the final day of the experiment there were no differences in percentage survival (Figure 4.4-3) ($F_{2,9} = 2.06$, $P = 0.18$), size heterogeneity ($F_{2,9} = 3.951$, $P = 0.059$) or specific growth rate ($F_{2,9} = 0.232$, $P = 0.798$) among treatments (Table 4.4-3). Also, there were no significant differences in any of the weekly bulk weights ($F_{8,45} = 0.353$, $P = 0.939$) (Figure 4.4-4).

Table 4.4-3 Survival, initial and final length, initial and final wet weight, size heterogeneity and specific growth rate (mean ± 1 S.E. of four replicates per treatment) of 21-day-old seahorses *H. abdominalis* cultured at three different stocking-densities in a 4-weeks growth trial.

Seahorses 3 l^{-1}	25	15	5
Final observed survival (%)	45.0±9.8	41.3±6.9	65.0±9.6
Initial individual weight (mg)	23.3±0.8	24.2±1.1	26.4±2.1
Final individual weight (mg)	56.8±1.8	55.4±3.2	61.4±4.2
Initial length (mm)	23.0±0.4	23.0±0.4	24.0±0.7
Final length (mm)	31.0±0.4	32.0±1.1	33.5±0.3
Size heterogeneity (body weight g)	1.37±0.40	0.73±0.10	0.52±0.10
SGR (% day^{-1})	2.86±0.20	2.65±0.30	2.73±0.20

Note: The use of superscripts has been omitted as there were no significant differences among treatments (a one-way ANOVA, $P < 0.05$).

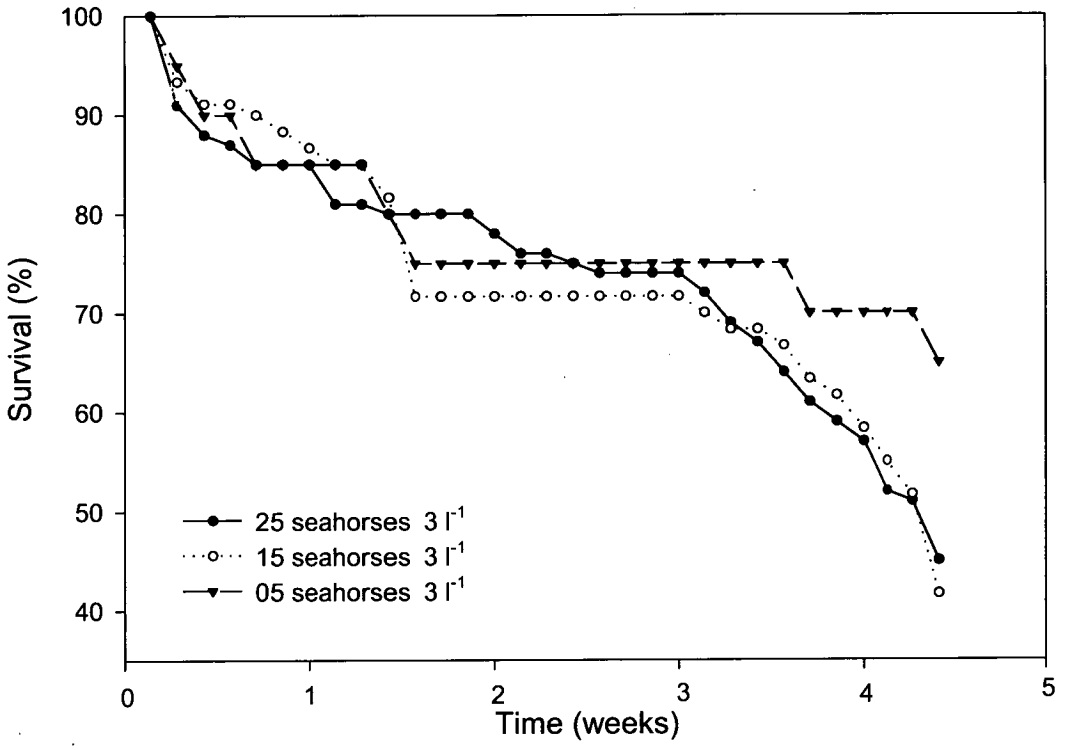

Figure 4.4-3 Survival of 21-day-old juvenile seahorses *H. abdominalis* cultured at three different stocking-densities over time. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

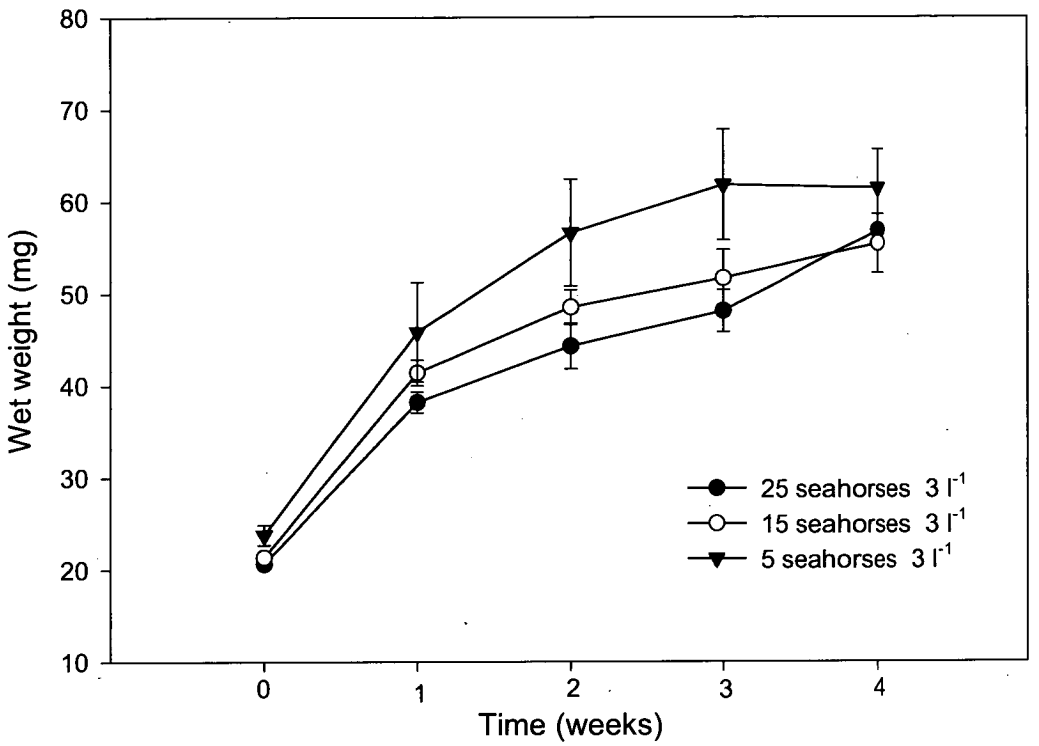

Figure 4.4-4 Wet weight of 21-day-old seahorses *H. abdominalis* cultured in three different stocking-densities over time. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment ± 1 S.E.

4.5 Discussion

Newborns and 21-day-old juvenile pot-bellied seahorses were grown at a number of stocking densities in this study. Such densities were lower than densities reported in the literature for other early stage marine finfish species (Daniels *et al.*, 1996; Baskerville-Bridges and Kling, 2000; Hatziathanasiou *et al.*, 2002) but higher than stocking densities tested on other seahorse species (Wong and Benzie, 2003; Woods, 2003b). They also were higher than the highest stocking density of 8 seahorses l^{-1} used in commercial scale systems for *H. abdominalis* during the first two months of their life (Martinez-Cardenas and Seahorse World Pty. Ltd. pers. obs.). Unfortunately the studies conducted by Wong and Benzie (2003) and Woods (2003b) used late juveniles which made comparisons difficult. Based on the results of the present study, the use of high stocking densities such as 45 seahorses 3-l^{-1} (15 seahorses l^{-1}) during the first two months of *H. abdominalis* life does not appear to significantly affect the growth compared to low stocking densities such as 5 seahorses 3-l^{-1} (1.6 seahorses l^{-1}).

The growth of fish at 45, 30 and 15 seahorses 3 l^{-1} reached a plateau after week five, while juveniles at 5 seahorses 3 l^{-1} increased linearly until the end of the experiment. Increases in stocking density magnify the competition for space and food, and also increases the concentration of ammonia, which is an indicator when the carrying capacity of a system is being overloaded (Alvarez-Gonzalez *et al.*, 2001). In this study, the samples for determination of water quality were taken from a randomly selected tank and unfortunately not from each tank; hence in-tank changes to water quality due to density were not monitored. However, the results of the colorimetric test indicated levels of ammonia, pH, nitrite and nitrates were suitable for *H. abdominalis* culture at all times (Adams *et al.*, 2001; Woods, 2005). While no studies have directly examined feeding hierarchies in seahorses, hierarchies would appear unlikely based on seahorse's general behaviour and dispersed prey availability in this study. However, more research is needed to examine the specific effect of stocking density on feeding behaviour. While feeding rates were standardised on the basis of BWd^{-1} the prey density would vary on the basis of fish density. While such a difference in food density may have affected capture success or competition for prey between fish, such measures were not undertaken in this study.

Perhaps the plateau displayed in growth between week five and week six could be explained by the greater amount of water added to the juvenile wet weight when they are measured in bulk in each tank (weeks two, three, four, and five) compared to the lesser amount of water added to juvenile wet weight when blot drying and measuring individual fish at the end of the experiment (as well as at the start of the experiment). In support of this assumption, growth recorded in juveniles at 5-seahorse l^{-1} appeared not to be affected by this issue, which could be a reflection of the similar amount of water added to the wet weight of seahorses when measuring in bulk and individually.

However, it is possible that the slowing of growth in the three treatments indicates a biomass limit for each tank, leading to a slowing of growth. In the second experiment this effect was not as evident as in the first experiment and the patterns were inconsistent with the first experiment. Although the measuring techniques used were the same as in the previous experiment the mean weight of the fish cultured at 5 seahorses 3 l^{-1} did not displayed the same increasing trend as in the first experiment. In contrast, the mean weight of the fish cultured at 25 seahorses 3 l^{-1} showed a continuous increase between week three and four despite being measured by two different techniques similar to the fist experiment and the organisms were larger than those used in experiment one. However, further research is needed to determine the biomass limit per tank during stocking density experimentation with early juveniles (less than 7 days-old).

The treatments displayed an overall survival rate of 59.0 ± 1.4 (mean ± 1 S.E.), which under the experimental conditions of this study represented a positive indicator for further research and commercial culture as one of the major constraints to the development of marine fish culture has been the successful rearing of fish beyond critical stages (i.e. first feeding). In most teleosts, chronic starvation of early stages produces low body condition, which easily leads to mortality (Shackley *et al.*, 1993; Tucker, 1998). Early stage mortality is also seen in seahorses including species such as *Hippocampus trimaculatus* and *Hippocampus kuda* where elevated mortalities occur at various critical stages, such as during the first 2-3 days of first feeding and after one week due to the change in prey-item/ prey-size from copepods to *Artemia* nauplii (Lin *et al.*, 2006; Sheng *et al.*, 2006). The inhibition of feeding during experiments with early juvenile *H. abdominalis* has also resulted in high mortalities (Woods,

2000a; Woods, 2000b; 2003a). Alternatively, the mortalities displayed in this experiment could be the result of inherent early juvenile mortality of *H. abdominalis*, or perhaps due to a negative response to a new environment resulting in a source of stress. This could affect seahorse growth beyond food ingestion, as there were no significant differences in the *Artemia* ingestion observations. The stocking-densities altered due to mortality and were not replaced, as replacement fish could not be identified through tagging or marking because of their small size. During the preparation for the first experiment, a range of tagging techniques were attempted: dorsal fin colouration with alcian blue, the fitting of a distinctive plastic collar around their neck and categorizing individuals by natural "body markers" such as slightly deformed tails as in Woods (2003b). Unfortunately, none of these attempts resulted in a reliable way to discriminate the replacements from the experimental fish. As replacement was not possible, the stocking densities of seahorses per each 3-l tank: 45, 30, 15 and 5, became on average: 30.0 ± 2.3, 20.0 ± 1.7, 10.0 ± 0.3 and 3.0 ± 0.5 (mean ± S.E.) respectively from day 13 until the end of the first experiment. Stocking-densities in the second experiment decreased from 25, 15, and 5 seahorses per each 3-l tank, became on average: 20.0 ± 0.7, 11.0 ± 1.6 and 4.0 ± 0.6 (mean ± S.E.) respectively.

Consistent with the first experiment, there were no significant differences among treatments in survival. In contrast, Woods (2003b) found that 5-month-old juvenile *H. abdominalis* survival was reduced and the physical interference among juveniles (i.e. tail grasping and wrestling) increased as a result of increasing the stocking-density from 1-2 seahorses l^{-1} to 5 seahorses l^{-1}. Wong and Benzie (2003) reported a lack of significant differences in growth of 3-month-old *H. whitei*, when testing a range of 0.5 to 1 seahorse l^{-1}. However, the authors acknowledged that this could be explained by the limited stocking density range tested.

In both experiments survival declined in a similar way during the first week. While mortality associated with the first feeding process may be an explanation for the first experiment it is unlikely in the second experiment suggesting that "early stage" mortality due to a deficient first feeding was not the only cause of seahorse mortality in the second experiment conducted with older fish. The causes of mortalities within the first week of culture/experimentation (despite the age of fish used) can be numerous and probably interrelated as suggested by Alvarez-Gonzalez *et al.* (2001). One possible cause of

mortalities could be related to the adaptability of seahorses to the experimental environment being transfer from a 20-l holding tank to the 3-l experimental tank. Hatziathanasiou (2002) found that European sea bass *Dicentrarchus labrax* larvae cultured at high stocking densities after being transfer to the experimental tanks developed negative social interactions leading to cannibalism. While seahorses do not display cannibalistic tendencies a negative social interaction has been reported by Woods (2000b) in which early stage seahorses in the absence of substratum formed "balls" of seahorses resulting in stress that easily lead to mortality as juveniles wrestled against each other instead of feeding. In the present study juveniles "balls" were not evident even at the highest stocking density, possibly because of the use of substrate.

In addition to swimming, seahorse behaviour includes the attachment with the tail to substrate during periods of inactivity and during the scotophase (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006). During these periods space availability is directly related to the availability of attachment substratum. In the present study, the attachment substratum was not a limiting factor as there were more nylon filaments than seahorses at the highest density of 45 seahorses per 3-l tank. Substrate was not provided proportionally to fish density but at the same level in all treatments. Substrate was not adjusted after the loss of fish. However, further research is needed to study the interaction between stocking density, and attachment availability. Aspects of substrate use will be explored in Chapter 8.

Post-handling stress (transport from Seahorse World Pty. Ltd. to the hatchery in the Aquaculture Centre at the University of Tasmania, Launceston) and poor adaptation-skills may explain to some extent the decline in survival during the first week of both experiments. Juveniles under stress are more likely to die after they reallocate metabolic energy from investment activities, such as growth, towards activities that require intensification to restore homeostasis (Bolasina *et al.*, 2006). In commercial situations the homogeneity of early juvenile production is desirable as the risk of disease or mortalities is reduced as it avoids the stress produced by grading and fish handling (Alvarez-Lajonchere *et al.*, 2002). In this study the homogenous sizes recorded in the weight of surviving seahorses was also reflected by the lack of significant differences in size heterogeneity across the treatments tested in both experiments. The adaptability of fish to a new environment and its size can vary when testing

high stocking-densities. King *et al.* (2000) found similar growth of summer flounder *Paralichthys dentatus* cultured at a range of stocking densities in a laboratory-scale system, while the results of testing the same stocking densities in commercial scaled trials showed a significant decrease in growth at the highest stocking densities.

In the commercial-scale culture of pot-bellied seahorse at Seahorse World Pty. Ltd. Beauty Point, the allocation of several broods in the same 50-l tank is a regular practice undertaken to optimise tank space. The stocking densities used in commercial *H. abdominalis* culture during early stages have been adopted without any scientific assessment. The results of this study provide information that could be used to optimize current practices. The stocking densities used in seahorse culture appear to be somewhat related to their reproductive strategy, which falls more under the K-selected description than pelagic fish as there is not an exogenous larval stage in seahorses. Seahorse growing culture starts after the release of relatively small numbers of well developed newborns from the male's pouch. In contrast, the stocking densities utilized with early stages of other commercial marine species are generally higher. Species such as Atlantic cod *Gadus morhua* (Baskerville-Bridges and Kling, 2000), southern flounder *Paralichthys lethostigma* (Odile *et al.*, 1996) and European sea bass *Dicentrarchus labrax* (Hatziathanasiou *et al.*, 2002) display a reproductive strategy more r-selected than seahorses. These species are generally cultured at high stocking densities despite the early mortalities due to the susceptibility of their endogenous-feeding dependent larvae. Therefore, the differences in the ontogenic development of seahorse compared with other species make it difficult to establish comparisons of the response to different stocking densities.

The stocking densities used in this study were higher than densities tested on *Hippocampus whitei* (Wong and Benzie, 2003), late juveniles of *H. abdominalis* (Woods, 2003b), and the highest stocking density of 8 seahorses l^{-1} used in commercial scale systems for *H. abdominalis* rearing during the first two months of their life (Martinez-Cardenas and Seahorse World Pty. Ltd., pers. obs.). To examine the effects of density on *H. abdominalis* in a commercial-size system, a large number of newborns would be needed in order to maintain the experimental stocking densities used in the first experiment (45, 30, 15, 5 seahorses 3 l^{-1}). Such a quantity of seahorses is not available to the author or is unlikely to be for any other

research facility for that matter. From the results of this study the author found that the use of high stocking-densities in early juveniles does not appear to compromise growth and may be adopted as an option to optimise the use of infrastructure and equipment under large-scale production. However, more research is needed to overcome the high mortalities recorded post-transfer in the experiments. The results in this study provide new and useful information from experimental scale trials. Future research is needed to analyse the effect of high stocking densities in the tank distribution of *H. abdominalis,* with an associated increase in substrate density in a pilot scale commercial trial.

4.6 Acknowledgements

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish. Ms Renee Florent, for her kind assistance on the improvement of earlier manuscript drafts and Mr Richard S. Wallace for the valuable information provided on commercial scale seahorse culture. The author was financially supported by Consejo Nacional de Ciencia y Tecnologia Mexico, with the additional support of Universidad Marista de Merida, Mexico.

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CHAPTER 5

EFFECT OF SALINITY ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

5 EFFECT OF SALINITY ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

5.1 Abstract

In Tasmania, commercial seahorse culture takes place in tank systems in which approximately 75 % of the water is exchanged daily from the Tamar River estuary. The aim of this study was to examine the effect on growth, condition, survival and *Artemia* ingestion on early juvenile pot-bellied seahorses *Hippocampus abdominalis* cultured at 5, 10, 15, 20, 25 and 32 ppt (g l^{-1}). The study focused on two main objectives: 1) to compare the effect of different salinities on fish directly transferred from 32 to salinities down to 5 ppt; and 2) to compare the effect of different salinities on fish after a gradual acclimation from 32 to salinities down to 5 ppt. Direct transfer of seahorses to 5 ppt resulted in 100 % mortality within seven days, although no evidence of poor condition or osmotic stress was detected in any treatment. An improvement in survival was recorded in seahorses direct transferred to 10 and 15 ppt compared to those at 25 and 32 ppt after nine days. During the 6-week trials seahorses were fed live enriched *Artemia* at a fixed rate of 14 % initial body weight day^{-1} (BW d^{-1}). Seahorses were able to grow, feed and survive similarly in a range of 15-32 ppt for 6 weeks. A temperature of 20 °C negatively affected growth at 15 ppt after 6 weeks of culture. After salinity acclimation, seahorses cultured over 6 weeks were able to survive in a range of 10-32 ppt. However, growth and condition was poorer at 10 ppt. Gradual acclimation did not improve survival in juveniles at 5 ppt, which recorded 100 % mortality after 48 h exposure. This study is the first to report on the salinity tolerance of *H. abdominalis*, showing that this species can be reared in captivity at a range of 15-32 ppt in early life stages without compromising growth and survival.

Keywords: *recirculation system; acclimation protocol; feeding activity; fish condition, whole body osmolality.*

5.2 Introduction

Full strength seawater has been commonly used during the experimental culture of species such as *Hippocampus erectus* (Correa *et al.*, 1989; Scarratt, 1996), *Hippocampus whitei* (Wong and Benzie, 2003) and *Hippocampus kuda* (Anil *et al.*, 1999; Lin *et al.*, 2006). Similarly, much of the research on the pot-bellied seahorse *H. abdominalis* also has been conducted in full strength seawater (Woods, 2000a, b; Adams *et al.*, 2001; Shapawi and Purser, 2003; Woods, 2003a, b, c; Woods and Valentino, 2003; Woods, 2005a,b; Wilson *et al.*, 2006). However, *H. abdominalis* is a coastal species found in estuarine waters of southeast Australia and New Zealand (Gomon and Neira, 1998; Martin-Smith and Vincent, 2005) and appears to tolerate water of fluctuating salinity (Foster and Vincent, 2004).

A significant number of pot-bellied seahorses inhabit the Tamar River estuary, which fluctuates significantly in salinity. Seahorse World Pty. Ltd. is located on this estuary. The company extracts and filters water directly from the estuary and has experienced salinities as low as 15 ppt. In general, little is known of the effect of salinity on seahorse physiology and production. Hilomen-Garcia *et al.* (2003) conducted a study on the salinity tolerance of *H. kuda* juveniles, in which growth and survival of this species improved when cultured in brackish water. However, that study was conducted on 63-day-old fish, providing a specific description for that life-stage. The adaptation of teleosts to hypotonic environments can vary with age (Smith *et al.*, 1999) hence the results found by Hilomen-Garcia *et al.* (2003) may be age- and species-specific.

Seahorse research has primarily been conducted on late juveniles as early stages present experimental difficulties such as higher mortalities compared to older fish. Not only can early juveniles be difficult to obtain, but there is also a limited quantity of tissue and blood available for early fish experimentation. Plasma osmolality is the measure of the ionic concentration in the blood which is affected by changes in water ionic content. In juvenile and adult teleosts the determination of plasma osmolality is used to measure the osmoregulatory adaptation of fish exposed to hypo- and hyper-osmotic environments. Maintenance of constant intracellular and extracellular ionic and osmotic conditions is critical for the normal functioning of cells. In fresh water environments fish must actively

take up salts, whereas in seawater they must excrete excess salts (McCormick and Bradshaw, 2006). Osmolality values higher or lower than those recorded within the natural salinity range at which the fish are generally exposed, reflect the ionic exchange (recorded by the osmometer) in the fish plasma as osmolality fluctuates in osmotically stressed fish.

In early stage fish the size limitation requires the use of whole-body osmolality instead of plasma osmolality (Tandler *et al.,* 1995; Moustakas *et al.,* 2004). In the present study the determination of whole-body osmolality was based on the technique described by Tandler *et al.* (1995) with some modifications due to the physical differences between gilthead bream (*Sparus aurata*) larvae, which present a softer body compared to the bony structure of juvenile seahorses. Another condition indicator used in early stage fish, as an alternative to proximate analysis is the determination of the moisture content (Fielder *et al.*, 2005) Elevated moisture levels can indicate a poorer condition in fish as fish exposed to sub-optimal conditions (i.e. exposure to water salinities outside the range generally experienced by the species) tend to replace muscle tissue with water as protein is utilized as an energy source to maintain homeostasis. This replacement starts when osmotically stressed fish cease food intake (Tucker, 1988); therefore *Artemia* ingestion rates can be an indicator of stress in fish exposed to salinities outside their natural range. The sodium: potassium ratio has also been considered an indicator of condition in early stage fish. Shackley *et al.* (1993) found a positive relationship between the sodium/potassium ratio and moisture content values, which were elevated while potassium decreased in starved fish. In the same study, the elevated values of moisture in starved fish were explained by the use of fat, proteins and carbohydrates being catabolized and then replaced by water. These techniques were used in this study in order to gain a better understanding of the response of early juvenile seahorses to various salinities.

Despite the previously mentioned seasonal fluctuations in salinity in northern Tasmania no studies on the effect of low water salinities have been conducted on any stage of the *H. abdominalis* life cycle. The use of low salinities in early stages of development has improved growth and survival of several species including turbot *Scophthalmus maximus* (Gaumet *et al.,* 1995), flounder *Rombosolea tapirina* (Hart *et al.,* 1996) and fat snook *Centropomus parallelus* (Rocha *et al.,* 2005). The development of a scientific protocol for seahorse culture

at different salinities could benefit commercial facilities as has already been done with temperature (Woods, 2001). However, the response of fish to certain salinities can vary at different temperatures (Santerre, 1976; Ottesen and Bolla, 1998).

The primary aim of this chapter was to determine the effect of salinities lower than 32 ppt in *H. abdominalis*. The two main aims in this chapter were: 1) to compare the effect of different salinities on fish direct transferred from 32 to salinities down to 5 ppt; and 2) to compare the effect of different salinities on fish after a gradual acclimation from 32 to salinities down to 5 ppt. Additionally the combined effect of a temperature of 20 °C with different salinities was examined, as the use of temperatures above 17 °C have been found to improve juvenile seahorse growth in Chapter Three.

5.3 Materials and Methods

5.3.1 System design and general methods

Juvenile pot-bellied seahorses were transported in seawater and oxygen-filled plastic bags inside an insulated container from a commercial seahorse farm (Seahorse World Pty. Ltd. Beauty Point, Tasmania) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. Experimental fish were reared from birth at a water temperature of 17 °C in 32 ppt (g l^{-1}) seawater. Following a 15-min temperature acclimation period the juveniles were allocated to a 20-l holding tank at the same conditions of birth. Four separate 35-l recirculation systems were used to maintain four salinities (Appendix one, diagram three). Each system comprised four 3-l tanks connected to a biofilter comprised of two stacked 22-l plastic containers. The upper container was filled with 40-mm bioballs and its floor area was perforated every five centimetres to allow the outflow water from the tanks trickle down to the container below. This lower container was used as a water reservoir in which a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) was installed. The pump provided an inflow of approximately 2.5 l hr^{-1} $tank^{-1}$ of 20µm-filtered seawater. In two reservoirs a heater was set to meet the desired temperatures for the corresponding sections of combined temperature and salinity. The other two systems met the desired temperature of 17 °C with the air conditioning set at that temperature.

Three litre tanks with static seawater were used for direct transfer experiments shorter than two week duration. The 30 % of the water volume of each tank was replaced in order to maintain good water quality. Water of different salinities was prepared by diluting seawater with dechlorinated tap water (0 ppt). A 12:12 (L:D) photoperiod was provided (lights on at 08:00 h, lights off 20:00 h) by a timer controlled cool white light 35 W (General Electric Company) producing an intensity of 4.8 $\mu E\ s^{-1}\ m^{-2}$ at the water surface. Continuous aeration was provided with flexible plastic tubing ending with a 4-l hr^{-1} plastic water-dripper (Neta®) acting as an air stone. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish. Attachment substratum for the fish was provided by a weighted bundle of 55 nylon monofilament segments with a length of 139.21 mm ±1.51 mm (mean ± 1 S.E.).

Water quality was follows: pH 7.78 (range 7.5-8.0), dissolved oxygen >75 %, total ammonia nitrogen (TAN) < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1}, nitrate < 5 mg l^{-1}. For the determination of pH, TAN, nitrite and nitrate, a colorimetric saltwater liquid test kit (Aquarium Pharmaceuticals Inc.) was used. Salinity and temperature were monitored every 24 h while TAN, pH, nitrite and nitrate were recorded every 48 h during the experiments. During the experiments the tanks were inspected daily for mortalities and any excess food and faeces were siphoned to waste.

The fish were fed live *Artemia* (enriched with Super Selco® for 24 hr at 17 °C) maintaining a rate of 14 % initial body weight day^{-1} (BW d^{-1}) (dry weight *Artemia*: wet weight fish) divided into two equal sized meals (10:00 and 16:00 h). *Artemia* fed at 16:00 h were from the same batch as the morning feed but were enriched for a further 6 h. Screens (150 µm) over the outlet of the tanks prevented the loss of *Artemia* during the day. Feeding time occurred from 08:00 to 19:00 h daily; at the end of this period the screens were replaced with 500 µm screens to flush out overnight into a central screen the remaining unenriched *Artemia*. Feeding adjustments were calculated based on the daily mortality (assigned the previously recorded mean weight) per tank in all trials (the rations corresponding to mortalities were not fed to the remainder of fish).Seahorse length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a 1 mm scaled sheet covered by plastic. Seahorse wet weight was measured on an analytical balance and recorded to the

nearest 0.0001g. In the long-term trials weekly growth was also recorded from bulk measures of wet weight. Fish were purged for 24 h before each weighing.

Fulton's K was calculated as $K = (W/L^3) \times 100$ where W = wet weight (g) and L = length (cm). Specific growth rate (SGR) was calculated as (SGR % increase in body weight day^{-1}) = $[(\ln W_f - \ln W_i)/t] \times 100$, where W_f = final weight (g), W_i = initial wet weight (g), and t = time (days).

5.3.2 Whole body osmolality and moisture content

Due to the small size of the seahorses it was not possible to obtain blood for plasma osmolality analysis. Whole body osmolality has been used as an alternative to plasma osmolality in early stage fish (Tandler *et al.*, 1995; Moustakas *et al.*, 2004) to determine osmotic stress. In this study whole body osmolality was determined using a technique modified from Tandler *et al.* (1995). Juveniles were euthanized with an overdose of benzocaine, blotted dry and their length and weight was measured. Seahorses were ground using a mortar and pestle adding osmosis-reversed water in a proportion of 1:10 (wet weight: water). The mix produced a homogeneous solution, which was transferred to a 1.5 ml Eppendorf tube and centrifuged for 7-min at 9000 rpm. The supernatant was transferred to a clean Eppendorf tube and stored frozen (-18 °C) until osmolality analysis using a Model 5520 VAPRO vapour pressure osmometer (Wescor, Inc., USA). One sample was taken from each tank (replicate) and read in the osmometer three times. The average was considered the value of one replicate for further analysis. The formula used in this study to calculate body osmolality was:

$C_b = (C_o [W*M + V_m] - C_m * V_m) / W * M$ (Tandler *et al.*, 1995)

Where C_b is body osmolality ($mOsmkg^{-1}H_2O$), C_o is the observed osmolality ($mOsmkg^{-1}H_2O$), W is fish sample wet weight (mg), M is body moisture (mg %), V_m is the volume of the dilution medium (µl), and C_m is the osmolality of the dilution medium.

After long-term trials one seahorse $tank^{-1}$ provided enough material for the determination of whole body osmolality. Due to the difference of sizes of seahorses analysed in the short-term trials, three seahorses were pooled in order to maintain the quantity of tissue diluted within a similar range. Sodium and potassium analyses were conducted using a fraction of the whole body supernatant in a Model SPECTRA AA 300 atomic absorption spectrometer (Varian, Inc. USA).

It has been found that in chronically starved fish a cellular re-arrangement on the content of sodium/potassium occurs. The body potassium (present in the intracellular space) decreases when body fat and body protein decrease. The intracellular space is replaced by extracellular fluid and the body sodium (located predominantly in the extracellular space) increases (Love, 1980). Based on the sodium and potassium content in seahorses, the ratio sodium/potassium was calculated as an additional measurement of body condition in experimental fish (Shackley *et al.*, 1993).

To determine moisture content, euthanized juveniles were wrapped individually in aluminium-foil and placed in an oven at 60 °C until constant weight was achieved. Moisture content was calculated using the following equation:

$$\text{Moisture} = ([W_w - W_d]) / W_w) \times 100.$$

Where W_w is wet weight (mg) and W_d is dry weight (mg).

5.3.3 Effect of direct transfer to low salinities

In Tasmania, the commercial scale culture of *H. abdominalis* experiences salinity declines. Therefore, the aim of the following trials was to determine the effect on growth and survival of juvenile seahorses transferred directly to salinities lower than full strength seawater (32 ppt). Initially a range of 5-32 ppt was selected to examine survival and behaviour (swimming, attached, lying on the bottom and floating on the surface); then specific analyses were conducted on the osmoregulatory response (whole body osmolality, moisture and body condition) of fish at 5 ppt, 10 ppt and 32 ppt. Based on those results, 6-week experiments

were conducted to examine the effect of a range of salinities (selected from those used in the short-term trials) on juvenile seahorses. Survival, whole body osmolality and moisture content were recorded for all treatments.

5.3.3.1 Exposure to low salinities over 4 and 9 days

Survival trial

The first trial was designed to examine a broad salinity range, used as an initial screening to identify the directions for subsequent trials. Three hundred and twenty-four 1-day-old juveniles from a single brood were used to determine the effect on survival following direct transferred to 5, 10, 15, 20, 25 (ppt) compared to juveniles maintained in a reference salinity of 32 ppt over a 9-day period. Eighteen juveniles were placed in each of 18 3-l transparent tanks (three replicates per salinity). In order to examine erratic behaviour as a stress indicator, seahorse activity 1 h after feeding was observed and recorded from a single observation per tank (three replicates per salinity) on day one and day nine. Four activity categories were identified: swimming, attached, lying on the bottom and floating on the surface (swim bladder hyperinflation).

Osmotic response trial

Based on the results of the previous trial, 630 1-day-old juveniles from a single brood were used in this trial. Thirty-five juveniles were randomly selected and directly transferred to each of nine 3-l tanks (three replicates per salinity) filled with water at three salinities: 5, 10 and 32 ppt. Two juveniles were randomly selected from each tank every 24 h; both juveniles were anaesthetized, euthanized and processed as previously described in section 5.3.2. One seahorse was used to determine whole-body osmolality and a second fish was used to determine its moisture content. The trial was conducted over four days. In all there were three fish per treatment for each analysis.

5.3.3.2 Exposure to low salinities over a 6-week period

Based on the previous experiments which indicated high survival rates over 4-9 days of exposure to low salinities, further studies aimed to contribute to a better understanding of the long-term implications of juvenile seahorse exposure to low salinities in commercial facilities. Two experiments were conducted to examine the effect of salinity on the survival, growth and *Artemia* ingestion of seahorses cultured over a long-term period (6-weeks) at salinities below full strength seawater after an initial direct transfer. The first trial focused on the response of juveniles to 15, 20, 25 32 ppt at the same temperature in which they were reared (17 °C) in the laboratory and in commercial culture (Seahorse World Pty. Ltd.). In addition, based on the results of the section on temperature, this study examined the interaction of salinity-temperature over a longer period. Therefore, a second experiment was conducted examining the response of juveniles to 20 °C and the four salinities previously described in the first experiment.

Effect of low salinities at 20 °C and 17 °C over a 6-week period

From a single brood of 325 juvenile seahorses, 240 1-day-old juveniles were used in the experiment at 17 °C after one day in a 20-l holding tank at 17 °C.

Juvenile *H. abdominalis* were randomly selected and transferred directly to low salinities in the experimental tanks. Fifteen juveniles were placed into each of sixteen 3-1 transparent tanks at 15, 20, 25, and 32 ppt at a constant 17 °C (16 tanks, four treatments with four replicates each). The length and wet weight of individual fish were recorded on day zero. After 6 weeks the surviving seahorses were counted and their weights and lengths were measured individually.

The second trial was conducted at a temperature of 20 °C. It is a common practice at Seahorse World Pty. Ltd., to combine various broods (2-3 days apart) in one 50-l tank in order to optimise space. Therefore, some of the subsequent experiments were conducted with combined broods.

Two hundred and forty juveniles seahorses were randomly selected from 750 fish from two broods (n = 290, n = 460; 4 and 3-day-old respectively). The seahorses were cultured and measured as described in the trial at 17 °C. Fifteen juveniles were placed into each of 16 3-1 transparent tanks at 15, 20, 25, and 32 ppt and a constant 20 °C (16 tanks, four treatments, four replicates).

On completion of each of the two experiments, four fish from each tank were randomly selected, euthanized and processed as previously described in section 5.3.2. Two of these juveniles selected were used for the determination of sodium, potassium and whole body osmolality analysis, while the remaining two seahorses were used to determine moisture content.

Effect of low salinities on Artemia ingestion

Low food intake can be a reflection of a poor osmoregulatory response to different salinities in stressed fish (Rubio *et al.,* 2005). To determine how the low salinities affected the food intake of juvenile *H. abdominalis*, *Artemia* ingestion was recorded during week one, three, and five in both experiments. A single fish was randomly selected per tank (replicate) and the number of feeding strikes produced during a 3-min period, 1 min after the food was introduced into the tank was recorded. One fish was recorded / tank /day over three days during each sample week.

5.3.4 Effect of gradual transfer to low salinities

After testing the effects of direct transfer on early juveniles *H. abdominalis,* it was suggested that a gradual transfer to lower salinities could improved their growth and survival. Commercial scale culture of *H. abdominalis* experiences salinity fluctuations during periods of high rainfall. Decreases of approximately 5 ppt generally occur due to tidal interaction (Seahorse World Pty. Ltd. pers. comm.). The aim of these trials was to determine the effect of gradual transfer from full 32 ppt to lower salinities in 5 ppt decrements. Initially short observations were conducted by examining the effect of the gradual decrease of salinity on

seahorse survival at two different temperatures: the reference temperature of 17 °C) and 20 °C, which was selected from Chapter Three. Then specific analyses were conducted on the osmoregulatory response of seahorses (whole body osmolality, moisture and body condition) throughout the salinity transfer at 17 °C. Based on those results, experiments were conducted to examine the effect of low salinities (selected from those used in short trials) in juvenile seahorses after gradual salinity transfer over a 6-week period. Survival, whole body osmolality and moisture content were recorded for all treatments.

5.3.4.1 Gradual transfer to low salinities over 11 and 12 days

Survival trial

The first trial was designed to examine the effect of gradual salinity transfer on seahorse survival at two different temperatures: the reference temperature of 17 °C and a higher temperature of 20 °C, which was selected from temperatures tested in the Chapter three. Two hundred and eighty eight 1-day-old seahorses from a single brood were used to determine the effect of salinity on the survival of the juveniles during the gradual salinity transfer for a period of 12 days. Eighteen fish were placed into each of 16 3-1 transparent tanks (four replicate tanks per treatment). Two recirculating systems (four tanks each) were used to hold juveniles in full strength seawater (32 ppt) at two temperatures (20 °C and 17 °C). The remaining two systems (four tanks each) were used to expose seahorses to a gradual salinity transfer from 32 ppt to 5 ppt at two constant temperatures (20 °C and 17 °C). For gradual salinity transfer, water was diluted every 48 h with fresh water to lower the salinity by 5 ppt decrements. Survival was recorded daily until the end of the trial on day 12.

In addition, based on the results of section 5.3.3.2, the subsequent trial examined the short-term effect of different temperatures on seahorses after a gradual salinity transfer. At the end of the gradual acclimation of trial one, four juveniles per tank were randomly selected from the full strength seawater (32 ppt) systems to examine the osmoregulatory response of seahorses at different temperatures. Three seahorses were randomly selected from each tank to be euthanized and processed as previously described for whole body osmolality in section 5.3.2. The seahorses used in this trial were approximately a third of the size of those used in

long-term trials. Therefore, in order to maintain proportions three seahorses were pooled together to form one sample. One additional seahorse was randomly selected from each tank to be euthanized and processed as previously described for moisture content in section 5.3.2

Osmotic response trial

Based on the results of trial one, trial two examined the osmoregulatory response of the juveniles during gradual salinity transfer. Four hundred 1-day-old seahorses from a single brood were used; fifty seahorses were placed into each of 8 3-l transparent tanks (four replicate tanks per treatment) for the 12-day trial. The same gradual salinity transfer used in the first trial was applied in this experiment except that it was conducted using a temperature of 17 °C instead of two different temperatures. Samples for whole-body osmolality and moisture content were collected 24 h after each dilution. Three seahorses were randomly selected from each tank to be euthanized and processed as previously described for whole body osmolality in section 5.3.2. As previously mentioned for trial one, the seahorses used in trial two were approximately a third of the size of those used in long-term trials. Therefore, in order to maintain proportions three seahorses were pooled together to form one sample. One additional seahorse was randomly selected from each tank to be euthanized and processed as previously described for moisture content in section 5.3.2

5.3.4.2 Gradual transfer to low salinities over a 6-week period

Two hundred and forty juveniles seahorses were randomly selected from 370 fish from three broods (n = 200, n = 50, n = 120; 5, 4 and 3-day-old respectively) produced at Seahorse World Pty. Ltd. Seahorses were exposed to 32, 15 and 10 ppt at a constant 17 °C to determine the effect of an acclimation protocol on long-term culture. Juveniles were gradually transferred from full strength sea water (32 ppt) to the salinities used in this experiment by sequential dilutions with fresh water every 48 h to lower the salinity by 5 ppt decrements. Fifteen juveniles were cultured over six weeks in each of 16 3-l tanks. The same culturing and sampling protocols described in section 5.3.3.3 were used in this experiment.

5.3.5 Statistical analysis

5.3.5.1 Survival and osmotic response trials

Seahorse activity (swimming, attached, lying on the bottom, floating) was compared in the initial and the final day of the trials using a χ^2 test of independence to determine if the proportion of juveniles in each category was the same across all salinities. Survival data was analysed using Kaplan-Meier survival analysis and a pairwise comparison over strata, with a Bonferroni correction of *P*-values. One-way analysis of variance (ANOVA) was used to compare the means of survival, whole body osmolality and moisture content among treatments at the end of trials.

After every one-way ANOVA Levene's Test and residual plots were used to test homogeneity of variance and Tukey's HSD post hoc tests were used to identify differences among treatment means (SPSS 11.5).

5.3.5.2 6-week trials

The means of initial length, final length (mm), initial weight, final wet weight (mg), survival (final day), whole body osmolality, sodium, potassium and moisture content (final day) were compared by one-way ANOVA among treatments. For *Artemia* ingestion observations an orthogonal ANOVA was used to compare the means of feeding strikes among treatments over the three-day observation (time as orthogonal factor) on each sample week. Levene's Test and residual plots were used to test homogeneity of variance. A natural-logarithm transformation was conducted on data of the sodium/potassium ratio in the experiment on long-term effect of low salinities at 17 °C in order to satisfy homogeneity of variance requirements. Tukey's HSD post hoc test was used to identify differences among treatment means, when using one-way ANOVA (SPSS 11.5).

5.4 Results

5.4.1 Effect of direct transfer to low salinities

5.4.1.1 Exposure to low salinities over 4 and 9 days

Survival trial

The Kaplan-Meier survival analysis in trial one showed significant differences among salinities ($P = 0.003$) (Figure 5.4-1). This result was consistent with the outcome of the ANOVA analysis of survival at the end of the 9-day trial. The percentage survival increased as the salinity decreased. More seahorses were able to survive in 10 ppt and 15 ppt than in 25 ppt and full strength seawater; survival at 20 ppt was similar to all groups ($F_{4,10} = 12.200$, $P = 0.001$) (Figure 5.4-1). In 5 ppt 100 % mortality was recorded on day seven.

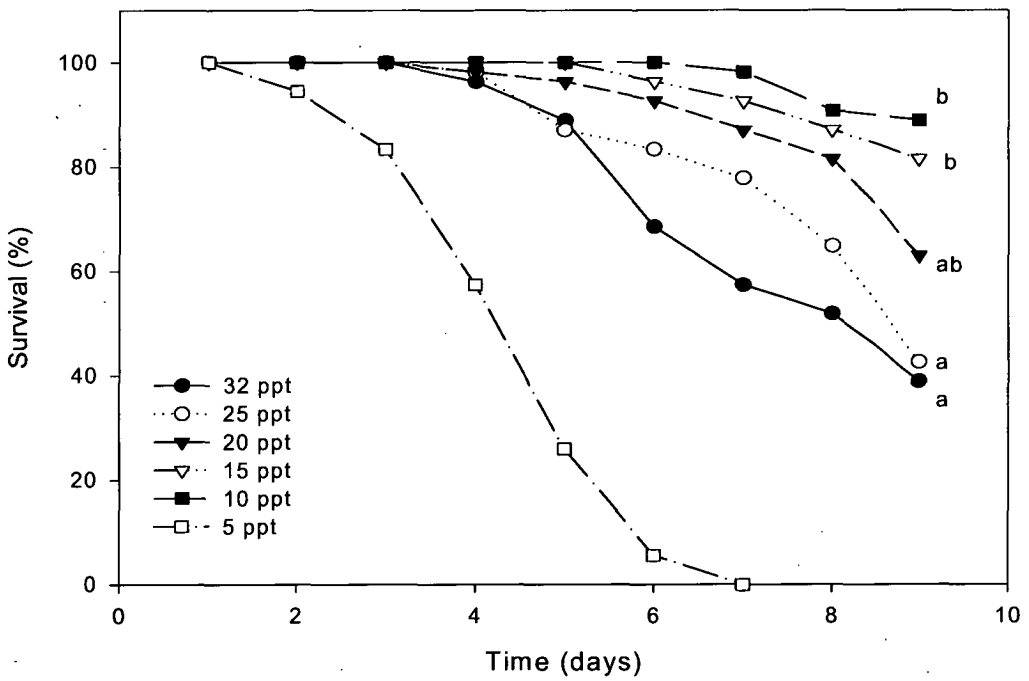


Figure 5.4-1 Survival of juvenile *H. abdominalis* after direct transfer from 32 ppt to five different salinities compared to a reference salinity of 32 ppt. All values represent the mean of three replicates per treatment. Different superscripts next to the final values indicate significant differences (ANOVA, $P < 0.05$). Standard error bars were omitted to aid visualization.

Table 5.4-1 Effect of three different salinities after direct transfer on wet weight, length, specific growth rate, Fulton's K, moisture and whole-body osmolality (means ± 1 S.E. of three replicates) of 1-day-old seahorses over a 4-day period.

Salinity	5 ppt	10 ppt	32 ppt
Initial individual weight (mg)	10.7±0.4[a]	10.0±0.6[a]	9.2±0.5[a]
Final individual weight (mg)	9.9±0.9[a]	12.4±2.1[a]	7.8±0.3[a]
Initial length (mm)	16.9±0.3[a]	16.7±0.3[a]	16.4±0.3[a]
Final length (mm)	17.1±0.6[a]	17.9±0.2[a]	16.9±0.1[a]
SGR (% day $^{-1}$)	-6.9±2[a]	0.6±2.8[a]	-7.2±5.4[a]
Condition (Fulton's K)	0.170±0.100[a]	0.170±0.008[a]	0.170±0.004[a]
Moisture (%)	85.1±1.6[a]	88.9±0.7[a]	88.0±0.8[a]
Whole body osmolality *(mOsmkg$^{-1}H_2O$)*	30.7±1.6[a]	38.8±3.6[a]	27.7±3.3[a]

Notes: Means with different superscripts within a row are significantly different (one-way ANOVA, $P < 0.05$).

The proportion of seahorses in each of the activity categories on day one was significantly different across all salinities ($\chi^2 = 90.17$, df = 10, $P < 0.001$). Swimming activity was the most frequent category across salinities except in 5 ppt (Figure 5.4-2). On day nine the proportion of seahorses in each behaviour category was similar across all salinities ($\chi^2 = 10.87$, df = 12, $P = 0.540$) (Figure 5.4-3).

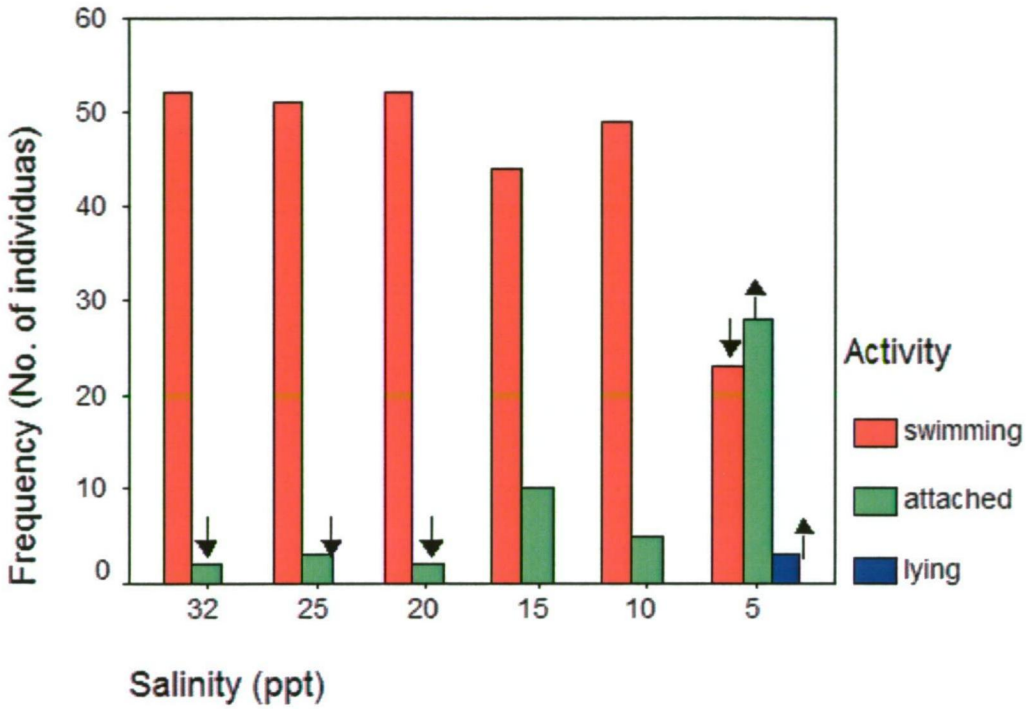


Figure 5.4-2 Effect of salinity on the activity of 1-day-old pot-bellied seahorses after direct transfer to five different salinities compared to a reference salinity of 32 ppt on day one. Data on swim bladder hyperinflation is not displayed as it was recorded as zero across salinities. Arrows are above cells that had normalized residuals (difference between observed and expected values) > ± 2.

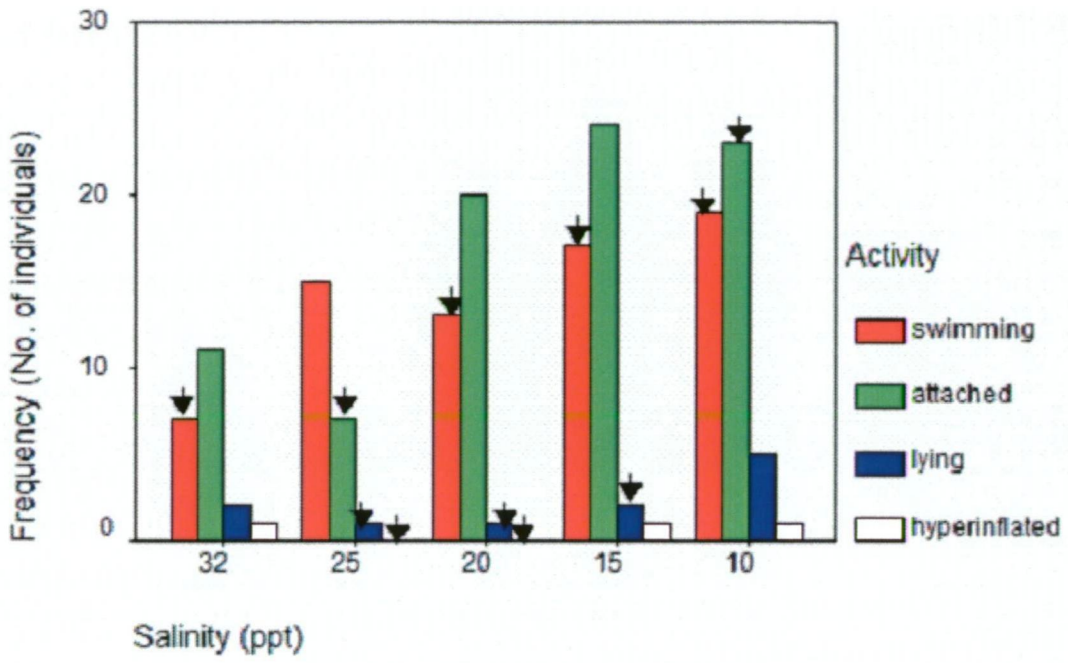
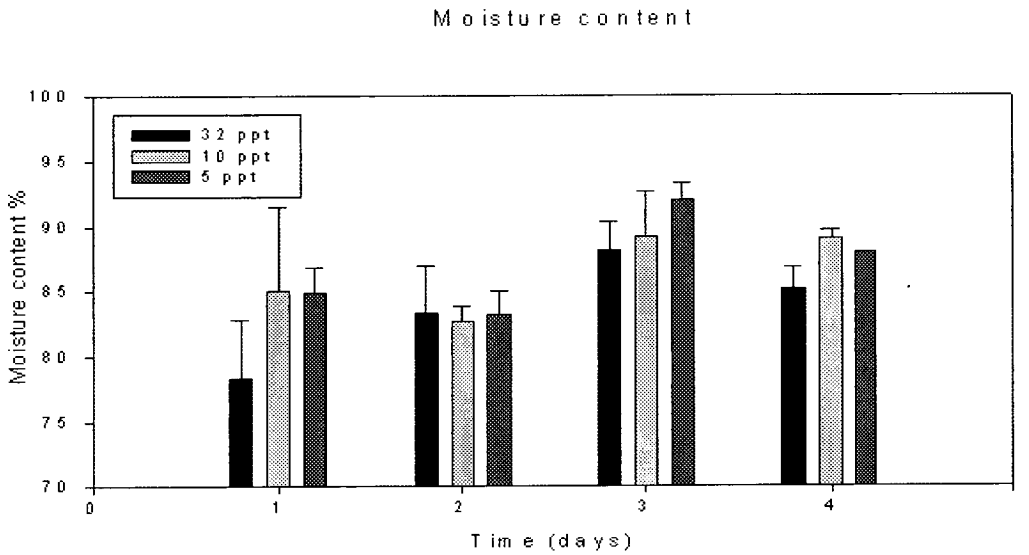


Figure 5.4-3 Effect of salinity on the activity of 9-day-old pot-bellied seahorses after direct transfer to five different salinities compared to a reference salinity of 32 ppt on day nine of the experiment. Arrows are above cells that had normalized residuals (difference between observed and expected values) > ± 2.

Osmotic response trial

At the start of the second trial, the juveniles did not show significant differences in length ($F_{2,33} = 0.582$, $P = 0.564$) or weight ($F_{2,33} = 1.675$, $P = 0.203$) among treatments. At the end of the trial (day four) no differences were recorded in length ($F_{2,29} = 2.57$, $P = 0.94$), wet weight ($F_{1,4} = 3.976$, $P = 0.093$), specific growth rate ($F_{2,29} = 1.247$, $P = 0.302$), or Fulton's K ($F_{2,29} = 0.169$, $P = 0.846$) (Table 5.4-1). There were no significant differences in moisture content ($F_{2,5} = 2.742$, $P = 0.157$) or whole body osmolality ($F_{2,4} = 3.702$, $P = 0.123$) of the juveniles across all salinities including 5 ppt until day four (Figure 5.4-4). At day five, 100 % mortality occurred in the tanks at 5 ppt.



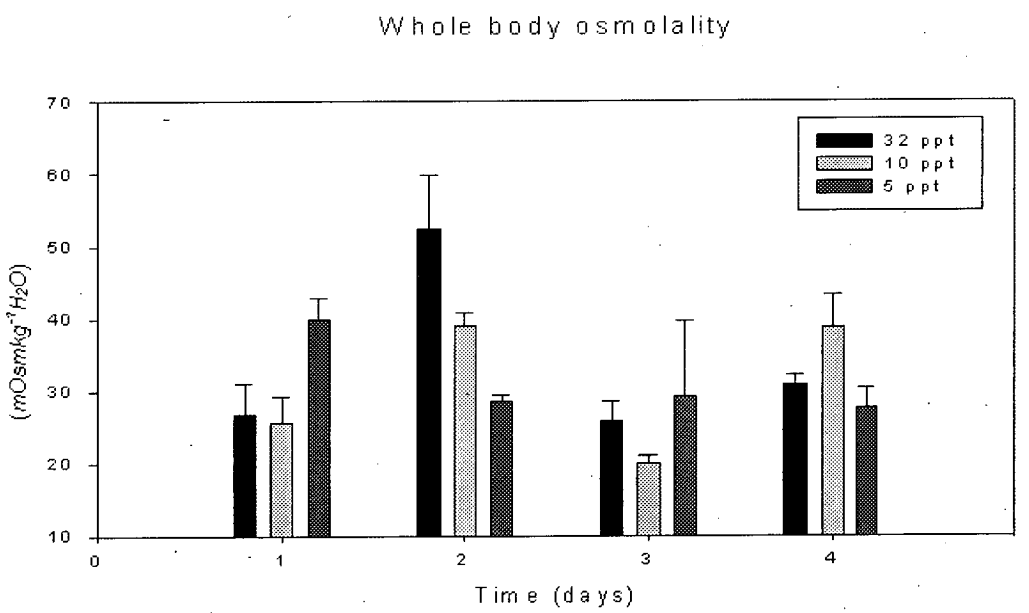


Figure 5.4-4 Moisture content and osmolality of 1-day-old pot-bellied seahorses after direct transfer to three different salinities for four days (mean ± 1 S.E. of four replicates per treatment). Moisture content was calculated as the difference in weight after oven drying (60 °C) expressed as a percentage of the initial wet (total) body weight. For whole-body osmolality three seahorses were homogenized in a proportion of 1:10 (wet-weight: osmosis-reversed water) for each replicate.

5.4.1.2 *Exposure to low salinities over a 6-week period*

Effect of low salinities at 17 °C

There were no significant differences in both length ($F_{3,12} = 0.65$, $P = 0.59$) and wet weight ($F_{3,12} = 1.770$, $P = 0.206$) among treatments at the start of the experiment (Table 5.4-2). After 6 weeks there were no significant differences in length ($F_{3,12} = 1.01$, $P = 0.42$), wet weight ($F_{3,12} = 1.61$, $P = 0.23$), Fulton's K ($F_{3,12} = 1.916$, $P = 0.181$), specific growth rate ($F_{3,12} = 2.760$, $P = 0.088$) or survival ($F_{3,12} = 1.69$, $P = 0.22$) of juvenile seahorses (Table 5.4-2, Figure 5.4-5).

There were no differences in whole body osmolality ($F_{3,8} = 1.04$, $P = 0.42$), sodium ($F_{3,8} = 2.248$, $P = 0.160$), or moisture content ($F_{3,12} = 1.41$, $P = 0.28$). A significant difference was detected by the ANOVA between the lower potassium content ($F_{3,7} = 5.27$, $P = 0.03$) of juveniles exposed to 15 and 20 ppt compared to the higher content recorded in those exposed to 25 and 32 ppt. There was also a significant difference between the higher sodium/potassium ratio of 15 ppt compared to that recorded in the higher salinities (25 and 32 ppt) ($F_{3,7} = 13.480$, $P = 0.003$) (Table 5.4-2).

Table 5.4-2 Effect of salinity after direct transfer on survival, wet weight, length, specific growth rate, Fulton's K, moisture, whole body osmolality, sodium, potassium and sodium/potassium ratio (mean ± 1 S.E. of four replicates per treatment) of 1-day-old pot-bellied seahorses cultured at four different salinities and 17 °C in a 6-week growth trial.

Salinity	15 ppt	20 ppt	25 ppt	32 ppt
Final observed survival (%)	45.0±12.0[a]	66.7±7.2[a]	51.7±5.7[a]	40.0±9.4[a]
Initial individual weight (mg)	9.2±0.3[a]	8.8±0.2[a]	8.5±0.1[a]	8.7±0.3[a]
Final individual weight (mg)	43.9±10.2[a]	52.5±6.5[a]	67.6±8.0[a]	60.9±7.3[a]
Initial length (mm)	17.00±0.10[a]	17.00±0.05[a]	17.00±0.03[a]	17.00±0.06[a]
Final length (mm)	30.0±2.7[a]	32.0±1.3[a]	35.0±1.4[a]	33.0±1.1[a]
SGR (% day $^{-1}$)	3.5±0.5[a]	4.2±0.3[a]	4.9±0.3[a]	4.6±0.2[a]
Condition (Fulton's K)	0.150±0.007[a]	0.150±0.003[a]	0.160±0.006[a]	0.170±0.005[a]
Moisture (%)	84.8±0.5[a]	84.2±0.1[a]	83.9±0.5[a]	83.3±0.6[a]
Whole body osmolality $(mOsmkg^{-1}H_2O)$	33.3±6.0[a]	23.9±3.0[a]	32.6±8.5[a]	37.5±0.4[a]
Sodium (μg/g/fish)	2847.9±488.0[a]	2263.7±349.0[a]	1864.8±6.2[a]	2110.8±127.0[a]
Potassium (μg/g/fish)	1334.9±3.0[a]	1339.0±111.0[a]	1782.0±113.0[b]	1699.0±86.0[b]
Sodium/potassium ratio	2.30±0.37[b]	1.60±0.12[ab]	1.05±0.06[a]	1.18±0.08[a]

Note: Mean values with different superscripts within a row are significantly different (one way ANOVA, $P < 0.05$).

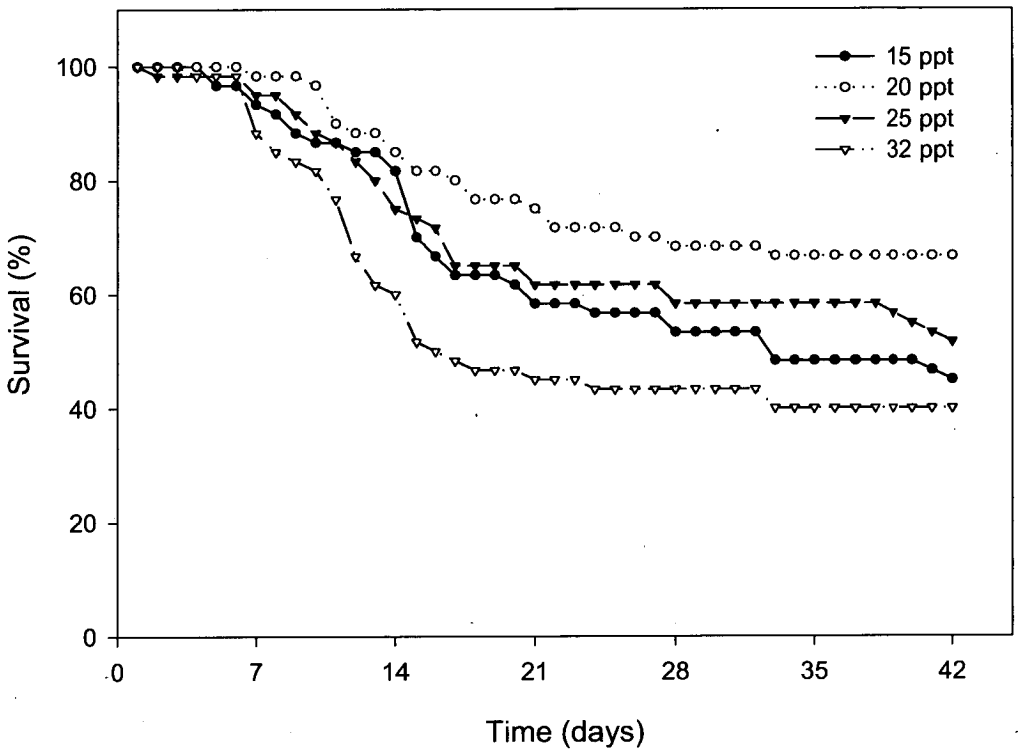


Figure 5.4-5 Survival of 1-day-old pot-bellied *H. abdominalis* cultured in four different salinities at a temperature of 17 °C in a 6-week growth trial. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

Effect of low salinities at 20 °C

There was a significant difference in length at the start of the experiment ($F_{3,12} = 4.45$, $P = 0.02$). However, the ANOVA detected at an accuracy of fractions of millimetres from the means, which is beyond the requirement of the methods proposed (nearest 1 mm). There were no significant differences in wet weight ($F_{3,12} = 1.49$, $P = 0.26$) among salinities at the start of the experiment (Table 5.4-3). After 6 weeks there were no significant differences in length ($F_{3,12} = 3.10$, $P = 0.06$) among treatments. Juveniles cultured at 15 ppt appear to be negatively affected by low salinity (15 ppt) especially in this experiment where the combination of a high temperature (20 °C) displayed a significant difference in juveniles at 15 ppt which grew less than those in the remaining salinities ($F_{3,12} = 5.55$, $P = 0.01$) (Table 5.4-3). Juveniles cultured at 15 ppt displayed a slower SGR than those at 25 ppt ($F_{3,12} = 4.550$, $P = 0.024$). There were no differences among treatments in survival ($F_{3,12} = 0.06$, $P = 0.97$), whole body osmolality ($F_{3,8} = 0.45$, $P = 0.72$), sodium ($F_{3,8} = 3.22$, $P = 0.08$), potassium ($F_{3,8} = 0.14$, $P = 0.93$), sodium/potassium ratio ($F_{3,8} = 1.342$, $P = 0.327$), Fulton's K ($F_{3,12} = 1.43$, $P = 0.28$) or moisture content ($F_{3,12} = 1.339$, $P = 0.308$). It was noted that the overall values of whole body osmolality were approximately double those recorded in the experiment at 17 °C (Table 5.4-3, Figure 5.4-6).

Table 5.4-3 Effect of salinity after direct transfer on survival, wet weight, length, specific growth rate, Fulton's K, moisture, whole body osmolality, sodium, potassium and sodium/potassium ratio (mean ± 1 S.E. of four replicates per treatment) of 3-day-old pot-bellied seahorses cultured at four different salinities and 20 °C in a 6-week growth trial.

Salinity	15 ppt	20 ppt	25 ppt	32 ppt
Final observed survival (%)	36.6±10.0^{a}	36.6±4.3^{a}	33.3±5.4^{a}	35.0±3.2^{a}
Initial individual weight (mg)	10.1±0.3^{a}	10.5±0.1^{a}	10.3±0.1^{a}	10.2±0.1^{a}
Final individual weight (mg)	82.2±13.0^{a}	137.6±8.9^{b}	143.3±17.4^{b}	132.9±4.5^{b}
Initial length (mm)	18.00±0.02^{ab}	18.00±0.03^{ab}	18.00±0.03^{a}	18.00±0.03^{b}
Final length (mm)	36.0±2.0^{a}	44.0±1.2^{a}	42.0±2.7^{a}	42.0±0.4^{a}
SGR (% day $^{-1}$)	4.5±0.5^{a}	6.1±0.2^{ab}	6.2±0.3^{b}	6.1±0.1^{ab}
Condition factor (Fulton's K)	0.160±0.005	0.160±0.009	0.190±0.024	0.170±0.008
Moisture content (%)	83.6±0.4^{a}	82.8±0.5^{a}	82.2±0.5^{a}	82.5±0.4^{a}
Whole body osmolality *(mOsmkg$^{-1}H_2O$)*	67.8±4.0^{a}	62.2±8.0^{a}	71.9±4.0^{a}	64.1±6.0^{a}
Sodium (μg/g /fish)	1895.2±11.0^{a}	2017.8±116.0^{a}	2235.4±19.0^{a}	2508.0±275.0^{a}
Potassium (μg /g/fish)	1804.3±87.0^{a}	1745.0±64.0^{a}	1805.6±51.0^{a}	1838.8±165.0^{a}
Sodium/potassium ratio	1.05±0.05^{a}	1.16±0.09^{a}	1.23±0.03^{a}	1.39±0.22^{a}

Note: Means values with different superscripts within a row are significantly different (one way ANOVA, $P < 0.05$).

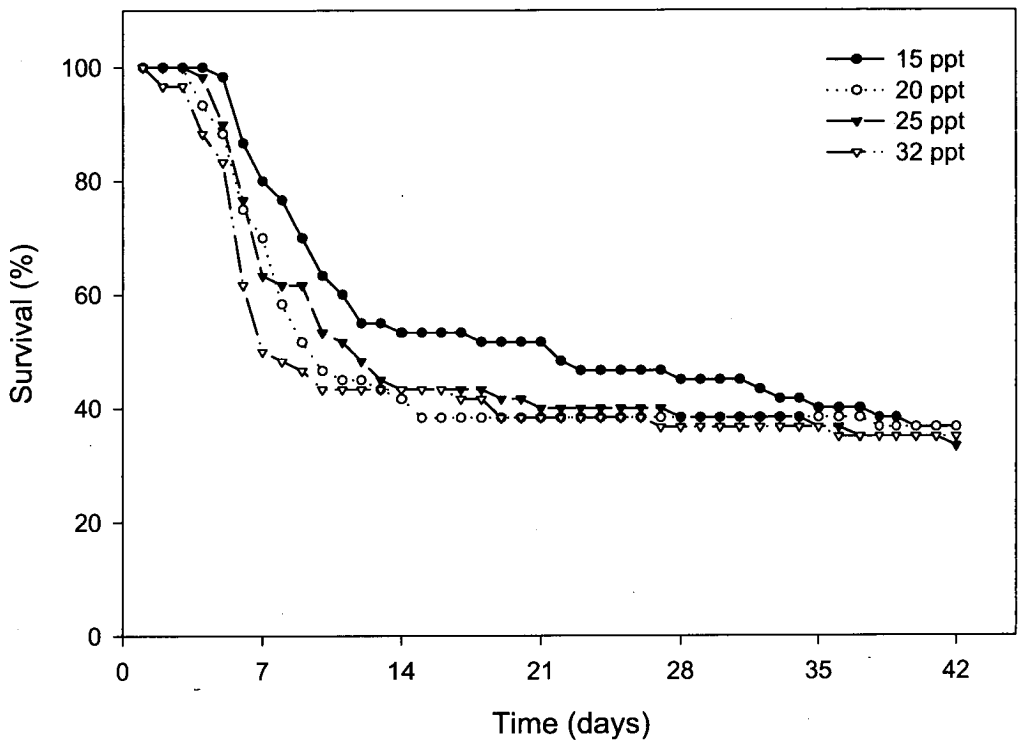


Figure 5.4-6 Survival of 1-day-old pot-bellied seahorses *H. abdominalis* cultured in four different salinities at 20 °C in a 6-week growth trial. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

5.4.1.3 *Effect of low salinities on Artemia ingestion*

Artemia ingestion was independent of salinity as there were no significant differences throughout the trial at 17 °C: during week one ($F_{6,36} = 1.503$, $P = 0.205$), week three ($F_{6,36} = 2.412$, $P = 0.046$) or week five ($F_{6,36} = 0.132$, $P = 0.991$) (Table 5.4-4). For the trial at 20 °C, there were no significant differences in either week one ($F_{6,36} = 0.238$, $P = 0.961$), week three ($F_{6,36} = 0.455$, $P = 0.836$) or week five ($F_{6,36} = 0.234$, $P = 0.963$) (Table 5.4-5).

Table 5.4-4 *Artemia* ingestion as strikes (mean ± 1 S.E. of four replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} in four different salinities and 17 °C, observed over three consecutive days in each of weeks one, three and five of the 6-week growth trial.

	Artemia ingestion								
Salinity ppt	Week one			Week three			Week five		
	Day one	Day two	Day three	Day one	Day two	Day three	Day one	Day two	Day three
15	10.3±3.1	25.0±5.9	14.3±2.6	12.5±3.8	26.0±4.6	19.8±3.5	26.3±9.8	15.0±3.3	16.3±5.6
20	20.3±5.2	22.3±4.1	12.3±2.3	16.5±5.5	17.8±4.9	15.0±5.0	22.5±12.6	7.0±3.2	11.8±5.4
25	9.0±2.4	25.8±6.4	19.0±5.8	15.0±1.3	11.3±1.6	20.8±4.6	18.0±4.4	8.0±5.7	13.5±6.4
32	11.0±1.9	13.8±5.8	17.8±2.5	29.5±6.2	24.5±9.6	7.5±5.0	21.8±4.5	15.0±6.7	12.8±3.5

Note: The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P < 0.05$).

Table 5.4-5 *Artemia* ingestion as strikes (mean ± 1 S.E. of four replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} in four different salinities at 20 °C, observed over three consecutive days in each of weeks one, 3 and five of the 6-week growth trial.

	Artemia ingestion								
Salinity ppt	Week one			Week three			Week five		
	Day one	Day two	Day three	Day one	Day two	Day three	Day one	Day two	Day three
15	22.3±4.0	19.8±5.6	16.0±6.0	16.5±4.3	17.3±8.2	9.3±2.8	19.3±12.9	25.0±9.2	18.0±7.7
20	35.8±8.0	26.0±3.9	19.8±3.8	22.8±5.3	25.5±3.8	23.3±6.3	23.3±7.8	23.5±2.3	20.0±3.8
25	31.8±7.5	21.5±3.9	21.3±3.3	28.3±5.8	22.3±5.2	24.5±2.7	31.3±6.9	24.3±7.1	22.3±3.3
32	29.0±6.9	21.8±5.3	23.0±7.2	20.0±4.6	23.8±8.3	10.5±5.8	17.0±3.0	18.5±4.0	20.5±7.9

Note: The use of superscripts has been omitted as there were no statistical differences among treatments (orthogonal ANOVA, $P < 0.05$).

5.4.2 Effect of gradual transfer to low salinities

5.4.2.1 Gradual transfer to low salinities over 12 and 11 days

Survival trial

The Kaplan-Meier survival analysis did not show any significant differences (Bonferroni correction of *P*-values; $P < 0.008$) among groups during the trial. On the last day (day 12) of the trial the ANOVA of survival showed no significant differences among treatments ($F_{3,12} = 1.671$, $P = 0.225$). Values from observations taken 48 h after exposure to 5 ppt were compared as after this time mortalities in the fish exposed to 5 ppt limited comparisons at 72 h of exposure. (Table 5.4-6). The comparison of juveniles held at 32 ppt at different temperatures did not show differences in whole body osmolality ($F_{1,6} = 0.043$, $P = 0.843$) or moisture ($F_{1,6} = 1.647$, $P = 0.247$) at the end of the trial (Table 5.4-6).

Table 5.4-6 Effect of salinity after gradual acclimation on survival, moisture and whole body osmolality of 4-day-old pot-bellied seahorses *H. abdominalis* exposed to constant 32 ppt at 17 and 20 °C compared to those exposed to decreasing salinities to the end point of 5 ppt at 17 and 20 °C. The moisture content and whole body osmolality were compared only between seahorses exposed to constant 32 ppt at 17 and 20 °C (mean ± 1 S.E. of four replicates per treatment).

Salinity	32 ppt	32 ppt	5 ppt	5 ppt
Temperature	17 °C	20 °C	17 °C	20 °C
Survival (%) after 48 h of exposure	66.6±6.0	77.7±3.9	62.4±3.5	70.7±6.2
Moisture (%)	85.4±0.8	84.3±0.2	-	-
Whole body osmolality $(mOsmkg^{-1}H_2O)$	42.0±2.5	42.6±1.4	-	-

The use of superscripts has been omitted as there were no significant differences among treatments (a one-way ANOVA, $P < 0.05$).

(-) Due to moisture and whole body osmolality were recorded after 72 h of exposure there was no data was recorded due to mortality.

Osmotic response trial

At the start the second trial there were no differences in length ($F_{1,4} = 0.364$, $P = 0.579$) or weight ($F_{1,4} = 2.861$, $P = 0.166$) among salinities. Based on the results of trial one, the final results of trial two were recorded on day 11 instead of day 12, in order to be able to record data from all the treatments tested, before high mortalities occurred, as indicated by trial one. There were no differences in wet weight ($F_{1,3} = 3.582$, $P = 0.155$), length ($F_{1,3} < 0.001$, $P = 0.1$), specific growth rate ($F_{1,3} = 7.499$, $P = 0.071$) or whole body osmolality ($F_{1,3} = 7.98$, $P = 0.06$) (Table 5.4-6). There were significant differences in moisture content with the juveniles in 5 ppt containing more water than the seahorse in 32 ppt ($F_{1,3} = 22.66$, $P = 0.01$). Fulton's K condition index was higher in seahorses at 32 ppt than the juveniles at 5 ppt ($F_{1,3} = 133.210$, $P = 0.001$) (Table 5.4-7).

Table 5.4-7 Effect of salinity after gradual acclimation on length, wet weight, specific growth rate, moisture, Fulton's K and whole body osmolality of 4-day-old pot-bellied seahorses *H. abdominalis* cultured in 32 ppt at 17 °C compared to juveniles cultured in an acclimation protocol at 17 °C in a 12-day-trial.

Treatments	Control	Acclimation
Initial	32 ppt	32 ppt
Initial Individual weight (mg)	8.9±1.4[a]	11.9±1.0[a]
Initial Length (mm)	16.6±0.8[a]	17.3±0.6[a]
Final length (mm)	24.0±0.0[a]	24.0±0.7[a]
Final individual weight (mg)	19.8±0.4[a]	16.0±1.5[a]
SGR (% day $^{-1}$)	6.4±1.5[a]	2.4±0.0[a]
Moisture (%)	87.2±0.5[a]	90.4±0.3[b]
Condition factor (Fulton's K)	0.190±0.003[a]	0.080±0.070[b]
Whole body osmolality *(mOsmkg$^{-1}H_2O$)*	58.9±3.6[a]	48.7±1.1[a]

Notes: Means with different superscripts within a row are significantly different (one way ANOVA, $P < 0.05$).

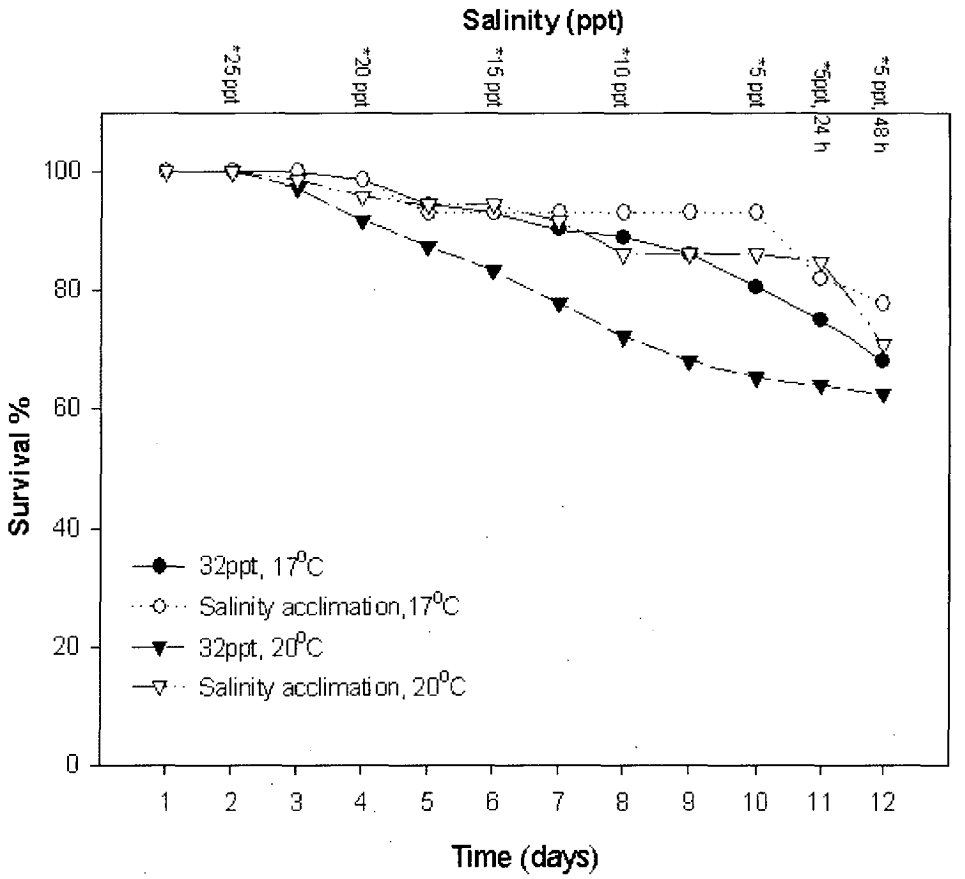


Figure 5.4-7 Survival of 1-day-old pot-bellied seahorses *H. abdominalis* cultured in 32 ppt at 20 °C and 17 °C (reference conditions) compared to juveniles cultures in an acclimation protocol at 20 °C and 17 °C. 100 % mortality was recorded in seahorses exposed to 5 ppt for 72 h in both temperatures. Asterisks indicate the salinity to which seahorses were exposed as the trial progressed. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

5.4.2.2 Gradual transfer to low salinities over a 6-week period

There were no significant differences in either length ($F_{3,12} = 2.55$, $P = 0.10$) or wet weight ($F_{3,12} = 0.312$, $P = 0.816$) among treatments at the start of the experiment (Table 5.4-8). After 6 weeks there were significant differences in length ($F_{2,9} = 4.89$, $P = 0.03$) and wet weight ($F_{2,9} = 8.01$, $P = 0.01$) as juveniles cultured in 10 ppt recorded a poorer growth compared to seahorses at 32 ppt. Juveniles cultured in 15 ppt did not show any differences in growth compared to the rest of the salinities. There were no significant differences on survival ($F_{2,9} = 1.122$, $P = 0.367$), whole-body osmolality ($F_{2,9} = 1.165$, $P = 0.355$), sodium ($F_{2,9} = 0.908$, $P = 0.437$), potassium ($F_{2,9} = 2.477$, $P = 0.139$), sodium/potassium ratio ($F_{2,9} = 1.88$, $P = 0.20$), SGR ($F_{2,9} = 3.579$, $P = 0.072$) or Fulton's K ($F_{2,9} = 0.535$, $P = 0.603$). The moisture content in seahorses cultured at 10 ppt was significantly higher compared to the remaining treatments ($F_{2,9} = 13.710$, $P = 0.002$) (Table 5.4-8, Figure 5.4-8).

Table 5.4-8 Effect of salinity after gradual acclimation on survival, wet weight, length, specific growth rate, Fulton's K, moisture, whole body osmolality, sodium, potassium and sodium/potassium ratio (mean ± 1 S.E. of four replicates per treatment) of early juvenile seahorses *H. abdominalis* cultured in three different salinities and 17 °C in a 6-week growth trial.

Salinity	10 ppt	15 ppt	32 ppt
Final observed survival (%)	38.0±3.2[a]	43.3±8.4[a]	30.0±5.8[a]
Initial individual weight (mg)	14.8±1.0[a]	14.1±0.5[a]	13.9±0.6[a]
Final individual weight (mg)	88.0±7.7[a]	131.2±9.8[b]	118.3±5.4[ab]
Initial length (mm)	20.0±0.3[a]	20.0±0.2[a]	20.0±0.4[a]
Final length (mm)	35.9±1.6[a]	41.6±1.5[b]	39.4±0.5[ab]
SGR (% day $^{-1}$)	4.28±0.02[a]	5.19±0.33[a]	5.05±0.03[a]
Condition factor (Fulton's K)	0.180±0.008[a]	0.180±0.009[a]	0.190±0.004[a]
Moisture (%)	86.0±0.8[a]	81.2±0.8[b]	82.1±0.2[b]
Whole body osmolality *(mOsmkg$^{-1}H_2O$)*	25.7±1.4[a]	28.5±1.3[a]	29.5±2.4[a]
Sodium (μg/g /fish)	1982±119[a]	1911±98[a]	2141±147[a]
Potassium (μg /g/fish)	1144.5±88.0[a]	1401.5±116.0[a]	1370.5±49.8[a]
Sodium/potassium ratio	1.77±0.20[a]	1.38±0.11[a]	1.55±0.05[a]

Note: Means with different superscripts within a row are significantly different (one way ANOVA, $P < 0.05$).

(-) No data was recorded due to mortality.

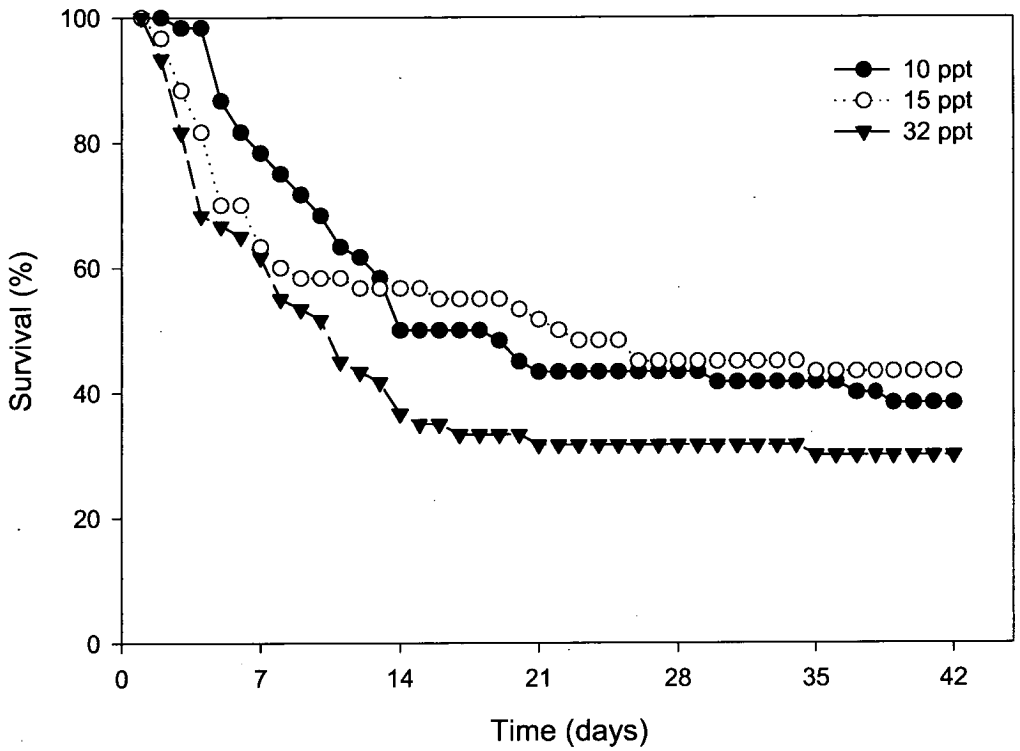


Figure 5.4-8 Survival of early juveniles *H. abdominalis* cultured in three different salinities in a 6-week growth trial. Seahorses were fed live *Artemia* at a ration of 14 % BW d^{-1} adjusted daily, based on growth and mortality. All values represent the mean of four replicates per treatment. Standard error bars were omitted to aid visualization.

5.5 Discussion

This chapter examined the gradual and direct transfer of juvenile *H. abdominalis* to a range of salinities potentially experienced by commercial facilities located in estuaries. Results indicate for the fist time that early juvenile *H. abdominalis* are able to grow in a range of 32 to 15 ppt while a salinity of 5 ppt was not tolerated producing 100 % mortality. The negative response of juveniles direct transferred to 5 ppt was reflected in their behaviour when, after 24 h of exposure the seahorses displayed less activity than the juveniles in the other salinities. At the end of the trial, after the loss of all juveniles in 5 ppt, fish in the remaining salinities exhibited similar patterns of activity (swimming) and inactivity (attaching).

The results from the second direct transfer trial suggested that early juveniles under commercial scale culture may survive short-term (e.g. 1 or 2 days) exposure to salinities as low as 5 ppt, if returned to salinities within the tolerance range (15-32 ppt). The results of adaptability to a hypotonic environment in this study indicate that *H. abdominalis* shares some similarities with *H. kuda.* Hilomen-Garcia *et al.* (2003) found that *H. kuda* juveniles survived at 10 ppt for a short period of time but they displayed an elevated moisture content after a longer period suggesting a reduced adaptability. In the present study, an improved survival was recorded in direct transferred 2-day-old seahorses when exposed for up to 9 days to 10 ppt and 15 ppt compared to those at 32 ppt.

The seahorses exposed to 5 ppt over a short period of time showed poorer condition as indicated by Fulton's K than those at a constant 32 ppt (control). Juveniles also showed an elevated level of moisture, which has been associated with poor adaptability (Hilomen-Garcia *et al.,* 2003). Moustakas et al. (2004) suggested that osmotically stressed larvae of *Paralichthys lethostigma* allocated energy to maintaining position thus leading to weakness. Westernhagen *et al.* (1998) similarly found that even short-term starvation can lead to loss of condition in fish. Therefore, the results suggest that once the seahorses were exposed to 5 ppt, they ceased feeding activity, leading to 100 % mortality in 72 h. Similarly, Rubio *et al.* (2005) found decreased food intake in sea bass *Dicentrarchus labrax* cultured at 7 ppt and 0 ppt compared to juveniles at 27 ppt.

In addition, while comparing the results of both short-term trials in this study (direct transfer and gradual transfer) it appears that direct transfer seahorses surviving for 144 h at 5 ppt were more tolerant than gradually transferred juveniles to 5 ppt which only survived up to 96 hours. Perhaps the reduced tolerance of the latter juveniles reflected the cumulative effect of the continuous salinity decrease until the end point (5 ppt) or it may reflect differences in the health status of each batch of juveniles.

Seahorses exposed to 10 ppt over 6 weeks after gradual acclimation to low salinities, displayed tendencies similar to those described by Shackley *et al.* (1993). In that study an elevated value of sodium/potassium ratio and an elevated amount of moisture content in starved fish indicated poor condition. In the present study, the amount of moisture in seahorses at 10 ppt was greater than the amount of moisture of seahorses at 15 ppt. Consistently a poorer growth was recorded in juveniles at 10 ppt compared to juveniles at 15 ppt suggesting the development of an associated poorer condition. As this effect was recorded only after six weeks of exposure, it perhaps indicates a reduced salinity tolerance range of *H. abdominalis* as seahorses grow. However, experimentation on the tolerance of late juveniles to lower salinities is required to better understand late juvenile adaptability. In contrast, Smith *et al.* (1999) and Varsamos *et al.* (2001) reported better adaptation to salinities lower than the salinity of seawater (35 ppt) in flounder *P. lethostigma* and sea bass *D. labrax* (respectively) as fish grew.

Juvenile *H. abdominalis* did not tolerate 5 ppt after being either gradually or directly transferred suggesting that beyond tolerance salinities the acclimation of early stages does not improve fish survival. Lethal effects of very low salinities have also been demonstrated in other marine fish. Smith *et al.* (1999) reported that early juvenile *P. lethostigma* exposed to fresh water resulted in similar low survival in either acclimated or non-acclimated groups compared to juveniles cultured in a salinity range tested of 5-30 ppt. However, *H. abdominalis* exposure to a salinity as low as 5 ppt in commercial culture would appear unlikely as such low salinities are not common, even during high rainfall periods due to the tidal contribution of seawater from the coast (Seahorse World Pty. Ltd. pers. comm.). In addition, Seahorse World extracts water from depths of 4-5 m to avoid mixture with freshwater at the surface. However such exposure may occur if seahorses were to be cultured

in seacages.

There was no apparent effect of salinity on growth and survival in juveniles cultured at salinities as low as 15 ppt at 17 °C over a 6-week period. However, the combination of low water salinity and a temperature of 20 °C resulted in a significantly poorer growth of juveniles cultured at 15 ppt compared to those at 25 ppt and 32 ppt. Despite the poor growth of fish in 15 ppt, Fulton's K values were not significantly different among treatments. Fulton's K has been used in seahorse research (Wong and Benzie, 2003) as an indicator of seahorse condition. In the present study it was expected that consistent with the poor growth of fish at 15 ppt, the condition recorded as Fulton's K in these seahorses would be also poor. However this was not the outcome. Future research is needed to determine the effects of the interaction between temperature and salinity on early juvenile seahorse condition.

The combination of low water salinity and higher temperature would appear unlikely to happen to early juvenile *H. abdominalis* in their natural environment as fluctuations in salinity occur due to rainfall runoff during high rainfall periods, which correspond to the winter-spring seasons in northern Tasmania. The opposite interactive effect of that seen in the present study has been reported in early juveniles of turbot *Scopthalmus maximus* (Imsland *et al.*, 2001) in which low salinities (15 ppt) combined with higher temperatures (23 °C) improved growth and food conversion efficiency. Growth of early stages of some species seems to be unaffected by the temperature-salinity interaction, for example as reported by Fielder *et al.* (2005) with snapper *Pagrus auratus*. In contrast, Hart *et al.* (1996) reported that survival rather than growth of flounder *Rhombosolea tapirina* was affected, when culturing larvae at 15 ppt compared to those at 25 and 35 ppt. Across the salinities tested (except 5 ppt due to mortality) fish were able to detect and ingest *Artemia* with no clearly consistent differences found among any particular salinity treatment.

Although comparisons have been made with other important commercial marine species, it must be noted that the response of teleosts to low salinities can be species-specific. The closest seahorse reference available is the work conducted by Hilomen-Garcia *et al.* (2003) on 9-week-old *H. kuda,* which appears to be more tolerant of low salinities than *H. abdominalis* as the authors reported survival of 65 % after 18 days following direct transfer

to 5 ppt.

Juvenile seahorses survived similarly at 10, 15 and 32 ppt for six weeks after being gradually transferred. Fielder *et al.* (2005) also reported no differences in survival in the culture of early stage snapper *P. auratus* in a range of 10-35 ppt. It was noted that during the three 6-week experiments overall survival displayed a decline in the first two weeks regardless of the duration of the transfer period (gradual, direct). However, there were no significant differences in survival at the end of any of the long-term experiments. As in previous chapters, it appears that during the first two weeks of the experimental period an inherent mortality affects seahorses across treatments. Those mortalities could be caused in part to stress related to the handling/measurement of the seahorses, or an adaptation period of juveniles to the experimental environment.

Growth and condition of seahorses in this experiment showed a negative response to 10 ppt. The results of Hilomen-Garcia *et al.* (2003) on *H. kuda* are similar to the present findings, in that juveniles can survive at 10 ppt for a short period of time. However, the elevated moisture content suggested reduced adaptability. Sampaio and Bianchini (2002) coincidently found growth depression in long-term (90 days) exposure to fresh water of flounder *Paralichtys orbignyanus,* and suggested an increase in energy expenditure associated with osmo- and iono-regulation under this salinity condition. Gaumet *et al.* (1995) in a study on S. *maximus* reported that turbot can tolerate 5 ppt in the short-term, but physiological parameters indicated that osmotic adjustments of turbot juveniles could be insufficient to allow complete adaptation (in the long-term) to a hypotonic environment.

The use of whole body osmolality instead of plasma osmolality was proposed for small size fish or early stages from which sufficient amounts of blood cannot be obtained. Whole body osmolality has been analysed from homogenized larvae samples of species such as sea bream (Tandler *et al.,* 1995) and flounder (Moustakas *et al.,* 2004). In the present study, none of the whole-body osmolality analysis showed a significant difference among treatments. This is contrary to the findings of Moustakas *et al.* (2004) where whole body osmolality of *Paralichthys lethostigma* larvae (15 days post-hatching) was higher at lower salinities (25 ppt) than at full strength seawater. Despite the author's efforts to maintain the consistency in

the homogenisation protocol (i.e. pooling three seahorses together in order to keep the dilutions within a similar range), the size and the body composition of seahorses processed from each experiment batch produced a different concentration in each experiment. This may also be explained by the variability while experimenting with different broods of fish. However, results were comparable within experiments, displaying the previously mentioned lack of significance. Fish in different broods may show a different response to environmental treatments but the use of one brood was not possible due to brood size. However, the response should be relative across treatments due to the mixed and random allocation of fish to tanks. The whole body osmolality samples from the long-term experiment at 20 °C were altered by incorrect storage, displaying overall values higher (approximately double) those in other experiments.

In the study conducted by Shackley *et al.* (1993) on starving salmon (*Salmo salar*) parr, the authors found a positive relationship between the sodium/potassium ratio and moisture content values, which were elevated in starved fish while potassium decreased. In the same study, the elevated amount of moisture in starved fish is explained by the fat, proteins and carbohydrates being used and catabolized and then replaced by water. In the present study, the lack of significant differences in growth may be explained in part by the adequate feeding ration provided to juvenile seahorses in contrast to the starvation periods experienced by salmon parr. However, the sodium/potassium ratio was clearly higher in seahorses cultured at 15 ppt than in those at 25 and 32 ppt and potassium was lower in 15 and 20 ppt. Although there were no significant differences in final body weight, Fulton's K or moisture content, the sodium/potassium ratio in seahorses cultured at 15 ppt may be an indication of the initial stages of a developing problem.

The salinity-temperature interaction during the six week trial showed a greater overall weight gain in juveniles cultured at 20 °C than seahorses cultured at 17 °C. These results are consistent with previous research by the author on temperature, where the growth of early stages of *H. abdominalis* was positively affected by temperature up to 23 °C over the control temperature of 17 °C (Chapter Three). In the present study after six weeks of culture at 17 °C and 20 °C the best weight gain was recorded in juveniles exposed to 25 ppt and the lowest in those at 15 ppt. In contrast, Hilomen-Garcia *et al.* (2003) reported that 15 ppt affected

positively the growth and survival of *H. kuda*. This may be explained by the better salinity tolerance to low salinities of *H. kuda* compared to that of *H. abdominalis*.

The overall weight gain recorded over six weeks in gradually transferred juveniles was greater than the weight gain of direct transferred seahorses cultured at 17 °C. Perhaps the gradual acclimation of juveniles to the salinities used in the experiments had a positive effect, while the negative effect due to a potential salinity shock produced by direct transfer to low salinities could be reflected in the poor seahorse growth after six weeks of culture. The present study is the first examination of the tolerance of *H. abdominalis* to low salinities. Early juvenile pot-bellied seahorses are capable of surviving in water salinities as low as 15 ppt without growth being compromised. A salinity of 10 ppt can cause poor fish condition in the long-term and a salinity of 5 ppt can lead to 100 % mortality within days. The effect of gradual acclimation suggests better growth compared to the growth of directly transferred seahorses. Commercial scale culture facilities such as Seahorse World Pty. Ltd may find these results useful when managing seasonal salinity fluctuations at their site. Further research would be useful to understand the effect at a physiological level (e.g. analysis of chloride cells, Na^+-K^+ - ATPase) of the salinities tested and whether seahorses are able to tolerate low salinities at low temperatures as experienced during winter rainfalls.

5.6 Acknowledgements

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish. Thanks to Ms Renee Florent, for her kind assistance on the improvement of earlier manuscript drafts and seahorse sampling. Also the author would like to thank Ms Maite Lijalad for her kind assistance during seahorse rearing and Mr Richard Scott Wallace for the valuable information provided on commercial scaled seahorse culture. The author was supported by CONACyT Mexico with the additional support of Universidad Marista de Merida.

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CHAPTER 6

EFFECT OF PHOTOPERIOD ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

6 EFFECT OF PHOTOPERIOD ON *ARTEMIA* INGESTION, GROWTH AND SURVIVAL IN CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

6.1 Abstract

Seahorses display depressed locomotor/feeding activity during the dark phase compared with the light phase. In order to determine potential growth benefits of using extended photoperiods in seahorse culture the effect of photoperiod on *Artemia* ingestion, growth and survival of early juvenile *Hippocampus abdominalis* was investigated in two experiments. Two different feeding rations were tested in the experiments. In the first experiment, in order to provide consistency of feeding across treatments, one meal (14 % initial dry matter of the body weight day^{-1}) was delivered at approximately 10:00 h to seahorses held under three photoperiod treatments: 24:00 (T24), 16:08 (T16) and 08:16 (T8) (L:D). *Artemia* ingestion, fish survival and body growth were recorded. At the end of the experiment, survival and growth in T16 and T8 were higher than in T24. However no differences were observed in *Artemia* ingestion. In the second experiment, early juvenile seahorses were cultured in the photoperiods and conditions described for the first experiment, except they were fed twice the amount of *Artemia* over two meals delivered at approximately 10:00 h and 16:30 h. At the end of the experiment the juveniles in T16 showed significantly better growth than the other two treatments, but there were no differences in survival. When food availability was not a limiting factor early juvenile seahorses grew better in an extended photoperiod such as 16:08 (L:D) but not in constant light.

Keywords: *recirculation system; continuous light; feeding activity; fish condition, food availability.*

6.2 Introduction

Exposure of early stages of fish to photoperiods longer than that of natural conditions has lead to an increase in growth rates in some commercial marine species such as snapper *Pagrus auratus* (Fielder *et al.*, 2002), gilthead sea bream *Sparus aurata* (Gines *et al.*, 2004), haddock *Melanogrammus aeglefinus* (Trippel and Neil, 2003), and red sea bream *Pagrus major* (Biswas *et al.*, 2005). *Hippocampus abdominalis* has been a useful seahorse model for aquaculture and biological research. As they are visual feeders, it is important to provide conditions to optimise prey ingestion to maximise growth and survival. Conditions which may influence this process include orientation of lights, light intensity and wavelength, contrast of prey against the background, tank colour, and photoperiod (Boeuf and Le Bail, 1999).

A major constraint in the development of marine fish culture has been the successful rearing of larvae beyond first feeding (Daniels *et al.*, 1996; Odile *et al.*, 1996). Seahorses do not display a conventional larval development like many other marine teleosts, with most development occurring in the pouch of the male prior to birth; therefore the newborns are considered early juveniles (Foster and Vincent, 2004). However, they do exhibit a prolonged "rearing" period of approximately four months during which they prey on live feed until a gradual transition from *Artemia* nauplii to frozen mysids takes place. *H. abdominalis* displays an ambush predatory strategy that can be affected by the interaction of the background colour and the prey pigmentation (Lovett, 1969; Karina *et al.*, 2006) and prey in-tank distribution (Woods, 2000). There are issues regarding food limitation during photoperiod experimentation on early stage fish, in which animals cultured under longer photoperiods expend more energy swimming and searching for food than the energy they assimilated by actually feeding (Fielder *et al.*, 2002; Gines *et al.*, 2004). One of the aims of this study was to examine, with the removal of any food limitation, the benefits (in body growth and survival) to early juveniles cultured under three different photoperiods.

As reported by Foster and Vincent (2004) most seahorse species display diurnal activity *in situ* with the exception of *Hippocampus comes* and *H. abdominalis* which displayed both diurnal and nocturnal activity. Typically, experimentation on seahorse husbandry has been

conducted with late juveniles (Hilomen-Garcia *et al.*, 2003; Wong and Benzie, 2003; Woods, 2003b; c), and very limited work has being undertaken on early developmental stages. Natural photoperiod regimes have been utilized in preliminary experiments on seahorse culture in order to simulate natural conditions; examples include the study by Wilson and Vincent (1998) on *Hippocampus kuda*, *Hippocampus barbouri* and *Hippocampus fuscus* and the study on *Hippocampus erectus* by Correa *et al.* (1989). In experimental husbandry of seahorses a 12:12 (L:D) photoperiod has been used for different species such as *Hippocampus whitei* (Wong and Benzie, 2003) and *Hippocampus subelongatus* (Payne and Rippingale, 2000). A 12:12 (L:D) photoperiod has been used in *H. abdominalis* experimentation in a range of studies, including the ammonia exposure test conducted by Adams *et al.* (2001), and the nutritional evaluations conducted by Shapawi and Purser (2003) and Wilson *et al.* (2006). Woods has covered several topics on *H. abdominalis* husbandry using a 12:12 (L:D) photoperiod in trials on stocking-density combined with gender segregation (Woods, 2003b), and diverse feeding strategies (Woods, 2003a; Woods, 2003c; Woods and Valentino, 2003). In addition, commercial scale facilities in Tasmania use a range of 12-13 h of light during the entire *H. abdominalis* life cycle (Seahorse World Pty. Ltd. pers. comm.).

Literature regarding the effect of different photoperiods on cultured seahorses has reported low activity during the dark period. Karina *et al.* (2006) conducted a study of late juvenile *Hippocampus reidi* in which adults were fed at dawn, noon, dusk and midnight. Zero feeding activity together with cessation of swimming at midnight was recorded. Similarly, Sheng *et al.* (2006) conducted a study on early juvenile *Hippocampus trimaculatus* and demonstrated that seahorses feed actively in the photophase but not during the scotophase. In the same study, the authors tested continuous feeding under a 24 h light regime recording lower feeding incidences during the period corresponding to night-time compared to the period corresponding to day-time. Ouyang (2005) fed late juvenile *H. abdominalis* continuously in a behavioural study while recording video images, and found significantly lower locomotor/feeding activity during the dark phase compared to the light phase. However, no study has been undertaken on the effect of photoperiod on the growth of early juveniles of *H. abdominalis*.

A study on *H. abdominalis* conducted by Woods (2000) reported that an increase of the photophase above 11 h promoted courtship behaviour in cultured *H. abdominalis* while a reduction from 11 h inhibits it, suggesting that mating is partially driven by the association with the day length in summer.

The effect of photoperiod on teleosts varies ontogenetically as younger fish have an inherently faster growth than older fish (Barlow *et al.*, 1995; Simensen *et al.*, 2000; Fielder *et al.*, 2002). This suggests a stronger response of early fish stages compared to older fish (El-Sayed and Kawanna, 2004). However, there are also reports on the negative effect of extended photoperiods as diverse sources of stress in marine fish culture, such as a negative social interaction leading to lower growth and survival caused primarily by cannibalism (Almazan-Rueda *et al.*, 2005) or the development of social hierarchies (Stefansson *et al.*, 2002) can be triggered by continuous light.

The overall aim of this research was to determine the effect of photoperiods extended /reduced from the photoperiod of 12:12 (L:D) used in standard culture practices on replicated groups of early juvenile pot-bellied seahorses *H. abdominalis* which will be exposed to one of three photoperiods: 24:00, 16:08 and 08:16 (L:D) over a 6-week period. The treatments 16:08 and 08:16 (L:D) were selected from the natural day length range in Tasmania, corresponding respectively to the maximum day length occurring in summer and minimum day length occurring in winter. Body growth, survival, and *Artemia* ingestion will be measured in order to determine the effect of different photoperiods on the intensive culture of this species. This study is the first to report on the effect of photoperiod on the growth of any seahorse species.

6.3 Materials and methods

6.3.1 System design and general methods

Juvenile seahorses used in the experiments were transported in 32 ppt (g l^{-1}) seawater and oxygen-filled plastic bags inside an insulated container from a commercial seahorse farm (Seahorse World Pty. Ltd. Beauty Point) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. After a 15 min temperature acclimation the fish were allocated to natural (fawn) coloured fibreglass 20-l holding tanks under a 12:12 (L:D) photoperiod until the start of the experiments.

Experimental 3-l tanks connected to a 100-l recirculation system were utilised. The recirculation system included a biofilter comprised of two stacked 40-l plastic containers. The upper container was filled with 40-mm bio balls and its floor area perforated every five centimetres to allow the outflow water from the tanks to trickle down to the container below. This lower container was used as a water reservoir in which was installed a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) that provided an inflow of 20µm filtered seawater at approximately 2.5-l hr^{-1} $tank^{-1}$. Continuous aeration was provided by flexible plastic tubing ending with a 4-l hr^{-1} plastic water-dripper (Neta®) acting as an air stone. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish. Attachment substratum for the fish was provided by a weighted bundle of 55 nylon monofilament segments with a length of 139.21 mm ±1.51 mm (mean ± 1 S.E.). Black plastic screening was installed around and above treatment tanks in order to isolate the three experimental photoperiods. Treatment tanks were positioned in blocks under each light. The illumination for each treatment was provided by 1 cool white light 35-W (General Electric Company) fluorescent tube producing an intensity of 4.8 µE s^{-1} m^{-2} at the water surface controlled by an inbuilt timer.

Water quality was maintained as follows: water temperature 17.3 °C (range 17.2-17.6 °C), pH 8 (range 7.5-8.4), salinity 32 ppt (range 31-33 ppt), dissolved oxygen > 75 %, total ammonia nitrogen (TAN) < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1} and nitrate < 5 mg l^{-1}. For the determination of pH, TAN, nitrite and nitrate, a colorimetric saltwater liquid test kit

(Aquarium Pharmaceuticals Inc.) was used. Salinity and temperature were monitored every 24 h while TAN, pH, nitrite and nitrate were recorded every 48 h during both experiments.

Tanks were inspected daily for mortalities and any excess food and faeces were siphoned to waste. Initial and final length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a submerged plastic-covered 1 mm scaled sheet. Initial and final wet-weight of seahorses, as well as the weekly bulk weights were measured on an analytical balance and recorded to the nearest 0.0001g. Fish were purged for 24 h before each weighing.

After 6 weeks, the surviving seahorses were counted and their individual weight and length were measured. The mean specific growth rate (SGR) for seahorses in each tank was calculated by SGR (% day $^{-1}$) = [(lnWf -lnWi)/t] x 100, where Wf = final weight, Wi = initial wet weight, and t = number of days. Also, in order to examine the development of size heterogeneity, the coefficient of variation (CV) of fish body weight (BW) was calculated (Kestemont *et al.*, 2003) followed by size heterogeneity = CV_{wf}/CV_{wi}; where $_{wf}$ = final weight, $_{wi}$ = initial wet weight, and CV = coefficient of variation (100 S.D. / mean). Elevated size heterogeneity on the basis of wet weight has been associated with the development of size-dependent hierarchies in experimental fish under extended photoperiod (Stefansson *et al.*, 2002). Although the seahorse literature has not reported any evidence of hierarchies, it was considered useful to examine the size heterogeneity as a growth response to the photoperiods used in this study.

6.3.2 Effect of photoperiod on growth and survival of early juveniles pot-bellied seahorses *H. abdominalis* in a 6-week trial fed one meal daily

From 300 juvenile seahorses from a single brood, 225 fish were used in this experiment. Five replicates of three photoperiod treatments were used: 24:00 (T24), 16:08 (T16) and 08:16 (L:D) (T8). In T16 and T8 lights were on simultaneously at 09:00 h. The 12:12 (L:D) photoperiod was not considered in this study as a reference treatment, as the results of using of 12-13 h of light have already proven to be effective in maintaining pot-bellied seahorses

culture (commercial and research) and did not fit the tank configuration available. Fifteen 2-day-old juveniles were randomly selected, and placed into each of the 15 3-l transparent tanks (five replicate tanks per each treatment). Their individual length and the wet weight were recorded on day zero. The fish were fed one meal daily at approximately 10:00 h with live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % initial body weight day^{-1} (dry weight *Artemia*: wet weight fish) with *Artemia* always present over the 24-h period. Weekly bulk weights were taken to determine increasing fish weight and to adjust the amount of *Artemia* needed to meet the feeding rate. The feeding rate therefore was adjusted throughout the entire experiment, on the basis of daily mortality (the rations corresponding to mortalities were not fed to the remaining fish) and weekly growth recorded from the bulk measures of wet weight per tank. Screens (150 µm) were placed over the outlet of the tanks to prevent the loss of *Artemia*. Each day, 1 h before feeding, the screens (150 µm) were replaced with 500 µm screens to flush out the remaining unenriched *Artemia*.

6.3.3 Effect of photoperiod on long-term growth and survival of early juvenile pot-bellied seahorses *H. abdominalis* fed two meals daily

Although *Artemia* was fed at a suitable rate in the previous experiment there was a concern that over the longer daylengths of T16 and T24 the density and enrichment levels of prey may have diminished and influenced fish growth rates. At the end of the photophase for T8 (aprox. 17:00 h) it was noticed that although there were nauplii in the tank most of the *Artemia* were in inaccessible areas of the tank (i.e. around screens). Therefore, after flushing the remaining *Artemia* at 16:15 h for a 15 min period a second meal of 14 % BW d^{-1} (dry weight *Artemia:* wet weight fish) was simultaneously provided to all treatments (at 16:30 h) at the rate previously described for the first experiment to avoid any possibility of food limitation especially for the extended photoperiod treatments.

Two hundred and twenty five juveniles were randomly selected from 460 juvenile seahorses from four broods (n = 170, n = 160, n = 80, n = 50; 4, 3, 2 and 1-day-old respectively) produced at Seahorse World Pty. Ltd. Seahorses were cultured under the three photoperiods

used in the first experiment. The same husbandry protocols were maintained with the exception of the feeding, in which the 14% BW was provided at approximately 10:00 h and 16:30 h (14% BW in each meal). Live *Artemia* fed at 16:30 h were from the same batch as the morning meal but were enriched for a further 6.5 h. After 6 weeks, the surviving seahorses were counted and their individual weight and length were measured.

6.3.4 Effect of photoperiod on *Artemia* ingestion

Direct visual observations of the number of feeding strikes (as measurement of *Artemia* ingestion) were undertaken during the first experiment. *Artemia* ingestion was recorded by randomly selecting a single fish per tank and counting the feeding strikes produced during a 3-min period, one minute after the food was introduced into the tank. Observations were undertaken on one seahorse $tank^{-1}$ day^{-1} over three consecutive days during week one and again during week five of the trial.

6.3.5 Statistical analysis

A one-way analysis of variance (ANOVA, SPSS 11.5) was used to compare means among treatments of initial length, final length (mm), initial weight, final wet weight (mg), survival and SGR of the seahorses cultured under the different photoperiods. Tukey's HSD post hoc test was used to identify differences among treatment means (SPSS 11.5).

An orthogonal ANOVA (SPSS 11.5) was used to compare the means of weekly (time as orthogonal factor) wet-weights among treatments throughout the experiment. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. Also an orthogonal ANOVA was used to compare the means of feeding strikes among treatments over the three-day observation in each sample week. A significance level of $P < 0.05$ was used. Levene's Test and residual plots were used to test homogeneity of variance. A natural-logarithm transformation was conducted on data of weekly weights in both experiments, in order to satisfy homogeneity of variance requirements.

6.4 Results

6.4.1 Effect of photoperiod on growth and survival of early juveniles pot-bellied seahorses *H. abdominalis* fed one meal daily in a 6-week trial

There were no significant differences in both length ($F_{2,12} = 0.87$, $P = 0.44$) and wet weight ($F_{2,12} = 1.74$, $P = 0.21$) among treatments at the start of the experiment. After 6 weeks, there were significant differences in length ($F_{2,12} = 12.530$, $P = 0.001$), and wet weight ($F_{2,12} = 12.290$, $P = 0.001$) among treatments (Table 6.4-1). Juveniles cultured in T24 grew less than those in T16 and T8; such growth was also reflected in the significant differences in bulk-weight among treatments throughout the experiment ($F_{2,84} = 5.148$, $P < 0.001$) (Figure 6.4-1). There were no significant differences in size heterogeneity ($F_{2,12} = 0.963$, $P = 0.409$) among the treatments. Survival of seahorses in this experiment showed a significant difference ($F_{2,12} = 4.58$, $P = 0.03$) between juveniles cultured in T24 and T8 (Figure 6.4-2) with higher survival recorded in T8. In T16, seahorse survival was intermediate to and similar to the other treatments (Table 6.4-1).

Table 6.4-1 Survival, initial and final length, initial and final wet weight, size heterogeneity and specific growth rate (mean ± 1 S.E. of five replicates per treatment) of 3-day-old juvenile pot-bellied seahorses *H. abdominalis* fed one meal daily (14 % BM day^{-1}) and exposed to three different photoperiods in a 6-week growth trial.

Photoperiod (L:D)	24:00	16:08	08:16
Final observed survival (%)	38.7±5.7^{a}	44.0±4.0^{b}	57.3±3.4^{b}
Initial individual weight (mg)	8.0±0.1^{a}	8.3±0.1^{a}	8.2±0.2^{a}
Final individual weight (mg)	53.7±2.7^{a}	86.6±7.3^{b}	86.1±5.1^{b}
Initial length (mm)	16.0±0.1^{a}	17.0±0.1^{a}	17.0±0.1^{a}
Final length (mm)	32.0±0.7^{a}	37.0±0.9^{b}	37.0±0.7^{b}
Size heterogeneity (body weight g)	2.8±0.3^{a}	3.0±0.4^{a}	2.3±0.3^{a}
SGR (% day $^{-1}$)	4.5±0.1^{a}	5.5±0.2^{b}	5.6±0.1^{b}

Note: Means with different superscripts within a row are significantly different (one way ANOVA, $P < 0.05$).

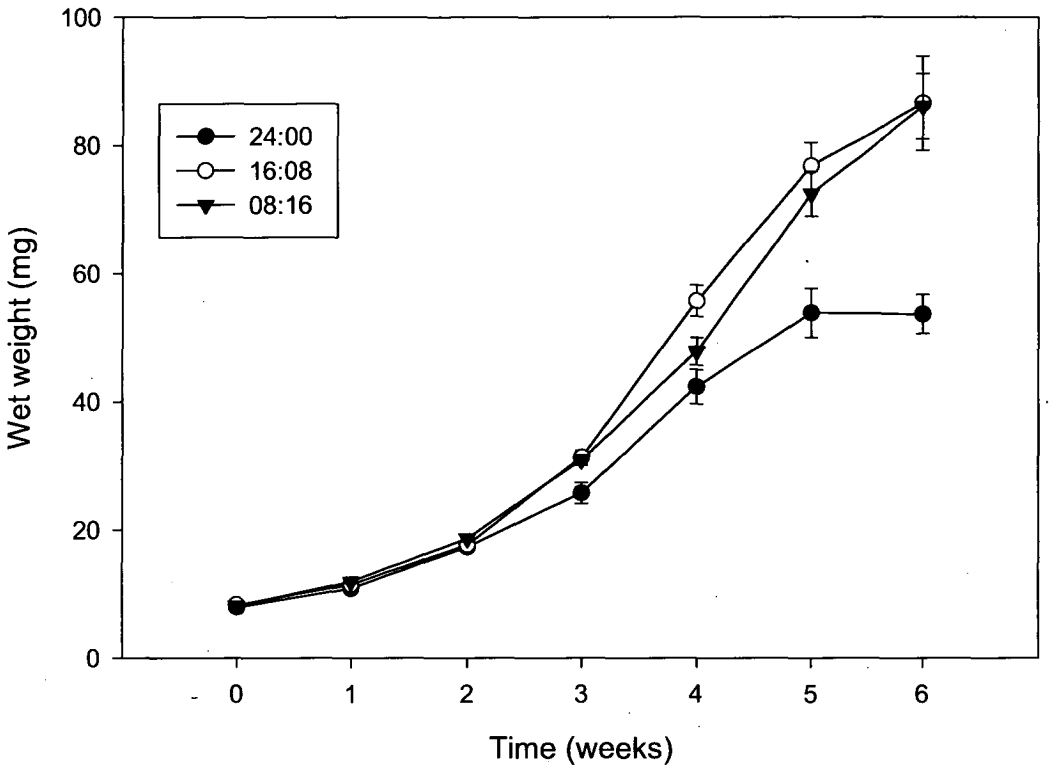

Figure 6.4-1 Wet weight of early juvenile pot-bellied seahorses *H. abdominalis* exposed to three different photoperiods in a 6-week growth trial. Seahorses were fed one daily meal of live *Artemia* at a ration of 14 % BW day^{-1} adjusted daily based on growth and mortality. All values represent the mean of five replicates per treatment ± 1 S.E.

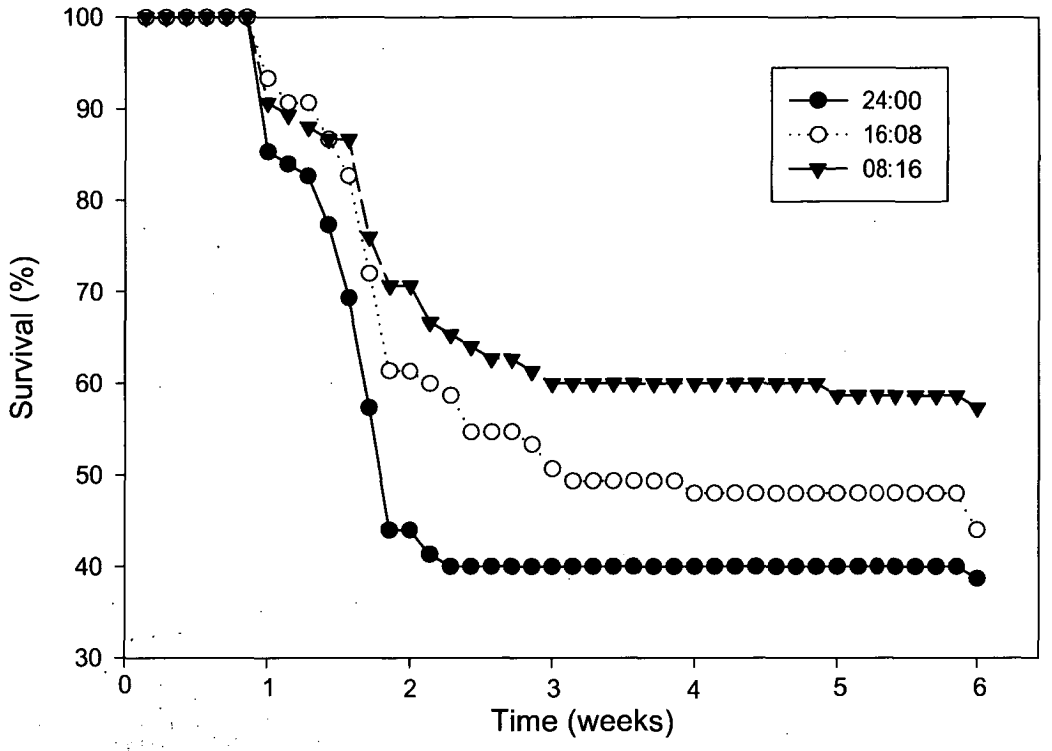

Figure 6.4-2 Survival of early juvenile pot-bellied seahorses *H. abdominalis* exposed to three different photoperiods in a 6-week growth trial. Seahorses were fed one daily meal of live *Artemia* at a ration of 14 % BW day^{-1} adjusted daily based on growth and mortality. All values represent the mean of five replicates per treatment. Standard error bars were removed to aid visualization.

6.4.2 Effect of photoperiod on growth and survival of early juvenile pot-bellied seahorses *H. abdominalis* fed two meals daily in a 6-week trial

There were no significant differences in both length ($F_{2,12} = 0.72$, $P = 0.50$) and wet weight ($F_{2,12} = 0.82$, $P = 0.46$) among treatments at the start of the experiment. After 6 weeks there were significant differences in length ($F_{2,12} = 8.150$, $P = 0.006$) and wet weight ($F_{2,12} = 10.350$, $P = 0.002$) where the juveniles cultured in T16 grew better than fish in T24 and T8 (Table 6.4-2). Such growth was also reflected in the significant differences in bulk-weight among treatments throughout experiment ($F_{12, 84} = 2.480$, $P = 0.008$) (Figure 6.4-3). There were no significant differences in size heterogeneity ($F_{2, 12} = 3.043$, $P = 0.085$) among the treatments. Survival of juvenile seahorses in this experiment showed no significant differences ($F_{2,12} = 2.71$, $P = 0.10$) among treatments (Figure 6.4-4).

Table 6.4-2 Survival, initial and final length, initial and final wet weight, size heterogeneity and specific growth rate (mean ± 1 S.E. of five replicates per treatment) of 3-day-old juvenile pot-bellied seahorses *H. abdominalis* exposed to three different photoperiods and fed twice a day (28 % BW day^{-1}) in a 6-week growth trial.

Photoperiod (L:D)	24:00	16:08	08:16
Final observed survival (%)	30.7 ± 4.5^{a}	42.7 ± 4.5^{a}	48.0 ± 6.8^{a}
Initial individual weight (mg)	11.0 ± 0.3^{a}	10.6 ± 0.3^{a}	10.5 ± 0.3^{a}
Final individual weight (mg)	98.2 ± 9.3^{a}	162.2 ± 11.4^{b}	107.7 ± 11.3^{a}
Initial length (mm)	18.0 ± 0.1^{a}	18.0 ± 0.2^{a}	18.0 ± 0.1^{a}
Final length (mm)	37 ± 1^{a}	42 ± 1^{b}	38 ± 1^{a}
Size heterogeneity (body weight g)	0.5 ± 0.1^{a}	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}
Specific growth rate (SGR % day^{-1})	5.2 ± 0.2^{a}	6.5 ± 0.2^{b}	5.5 ± 0.3^{a}

Note: Means with different superscripts within a row are significantly different (one-way ANOVA, $P < 0.05$).

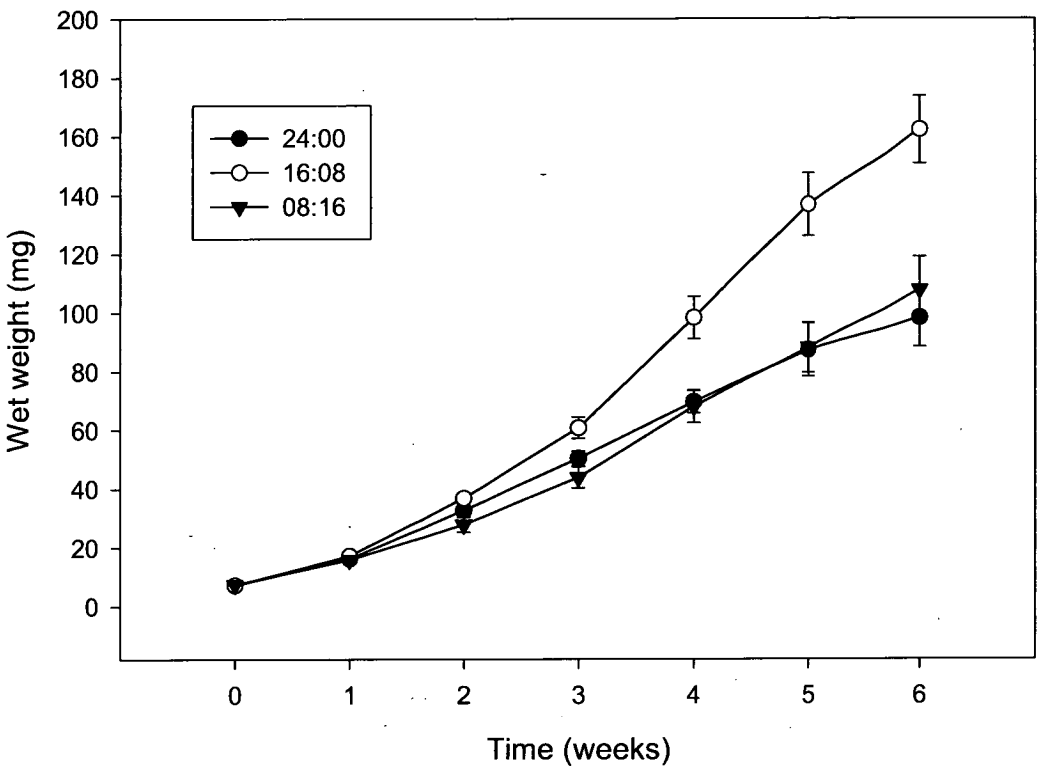

Figure 6.4-3 Wet weight of early juvenile pot-bellied seahorses *H. abdominalis* exposed to three different photoperiods in a 6-week growth trial. Seahorses were fed live *Artemia* twice a day at a ration of 28 % BW day^{-1} adjusted daily based on growth and mortality. All values represent the mean of five replicates per treatment ± 1 S.E.

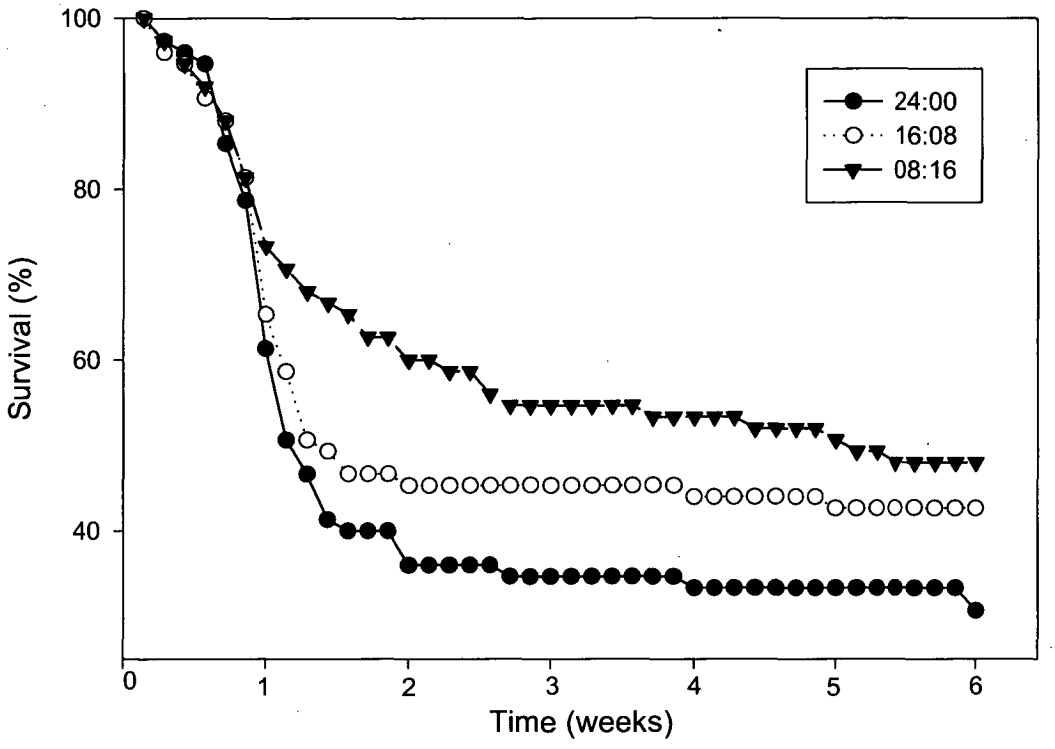

Figure 6.4-4 Survival (% mean) of early juvenile pot-bellied seahorses *H. abdominalis* exposed to three different photoperiods in a 6-week growth trial. Seahorses were fed live *Artemia* twice a day at a ration of 28 % BW d^{-1} adjusted daily based on growth and mortality. All values represent the mean of five replicates per treatment. Standard error bars were omitted to aid visualization.

6.4.3 Effect of photoperiod on *Artemia* ingestion

There were no significant differences in *Artemia* ingestion throughout experiment, in either week one ($F_{4,36} = 1.036$, $P = 0.402$) or week five ($F_{4,36} = 0.763$, $P = 0.556$) (Table 6.4-3).

Table 6.4-3 *Artemia* ingestion as strikes (mean ± 1 S.E. of five replicates per treatment) over a 3-min period recorded from one randomly selected fish tank^{-1} in three different photoperiods in a 6-week growth trial

	Feeding frequency					
Photoperiod (L:D)	Week one			Week five		
	Day one	Day two	Day three	Day one	Day two	Day three
24:00	22.0±2.6	8.2±4.2	23.0±4.2	5.4±1.1	7.2±5.0	3.6±1.1
16:08	16.4±4.3	9.8±3.5	23.0±6.3	15.0±4.3	5.0±2.2	9.2±5.2
08:16	24.4±4.8	21.8±5.3	22.0±2.3	14.4±4.2	14.2±3.1	15.8±5.1

Note: The use of superscripts has been omitted as there were no significant differences among treatments (orthogonal ANOVA, $P < 0.05$).

6.5 Discussion

The present study is the first to report the effect of photoperiod on growth and survival of early juvenile pot-bellied seahorses *H. abdominalis*. The absence of improved growth and survival in seahorses exposed to 08:16 (L:D) is consistent with the literature, which suggests the inactivity of cultured seahorses during the scotophase (dark period) as well as their possible inability to see their prey in the dark (Ouyang, 2005; Karina *et al.,* 2006; Sheng *et al.*, 2006) impacts on food intake. Similarly the negative impact of the 24 h regime on seahorse growth and survival suggests that this photoperiod might not provide any benefits for commercial scale culture.

The fish cultured in T24 in the first experiment showed the lowest growth and survival. As there was a possibility that the adjusted feeding rate of 14 % body weight day^{-1} (dry weight *Artemia*: wet weight fish) and the quality of the *Artemia* after a certain period of time was not appropriate to compensate the energy expended by the seahorses cultured under the 24 h photoperiod, the feeding strategy was altered in the second experiment. However, it would appear unlikely that a feeding rate of 14 % body weight day^{-1} would be limiting, as a daily feeding rate of 5 % of body weight has been used previously for *H. abdominalis* experimentation on newborns (Florent, 2003), juveniles (Wardley, 2001; Wilson *et al.,* 2006) and late juveniles (Woods, 2005). The feeding rate of 14 % body weight day^{-1} utilized in this study was based on its use in previous chapters. In those trials the rate used was found to be in excess for early juvenile *H. abdominalis* (Chapter Two).

In the present study, although there were *Artemia* in the tanks at all times, not all the live food was available as some nauplii tended to congregate in inaccessible areas of the tanks (i.e. over the filtering screens and near to the surface and walls). Also the nutritional value of the *Artemia* remaining in the tanks over the 24 h feeding cycle could be low due to evacuation of the enrichment media by the nauplius digestive system. Previous work on juvenile seahorses has shown that the use of unenriched *Artemia* to support good growth and survival of juvenile *H. abdominalis* is not recommended (Shapawi and Purser, 2003). In addition, other studies have found possible stress effects associated with continuous light (Stefansson *et al.,* 2002; Turker, 2005) and this may be a cause of the poor growth in T24 in

this experiment.

Seahorses cultured under adequate water conditions display eager feeding behaviour (Woods, 2000). An extended photoperiod (T16) did not appear to be a good condition for juveniles cultured in the first experiment as it did not promote any improvement in growth or survival compared to those in T8. Seahorse feeding activity is primarily displayed during the light period as reported by Ouyang (2005) for late juvenile *H. abdominalis*, Karina *et al.* (2006) for late juvenile *H. reidi*, and Sheng *et al.* (2006) for early juvenile *H. trimaculatus*. These studies help explain to some extent the suggestion that the *Artemia* remaining in tanks under 08:16 (L:D) in this experiment may not have been ingested during the scotophase and that 8 h was a sub-optimal feeding duration. This was substantiated by direct observation of excess *Artemia* in the tanks of this treatment. Based on the same literature reports, the juveniles in T16 in this experiment should benefit from the extended feeding period, but they did not in the first experiment. Trippel and Neil (2003) found that an extended feeding period for juvenile haddock *Melanogrammus aeglefinus* did not enhance growth under equivalent rations, suggesting that haddock may be capable of reaching satiation during a feeding period equivalent to natural day length. Seahorses in T24, T16 and T8 were fed at the same time (at approximately 10:00 h) and at the same feed rate based on %BW adjusted for daily mortality and weekly growth recorded in the bulk measures of wet weight per tank. However, it is possible that the quality of the *Artemia* available for the fish in T16 was not adequate towards the end of this photoperiod, or that a proportion of the *Artemia* was not available to the fish because of their concentration over the filtering screens and near to the surface and walls.

To avoid any confounding influence from food limitation and the effect of declining nutritional value of *Artemia* due to evacuation of enrichment, feeding was doubled in the second experiment compared to the ration used in the first experiment. At the end of the experiment, juveniles under the extended photoperiod treatment (T16) showed the highest growth, suggesting that the quantity and quality of the food (as *Artemia* fed at approximately 16:30 h was also maintained in enrichment media prior to feeding) was more appropriate for growth of juveniles cultured in T16 compared to seahorses cultured in T16 during the first experiment.

Survival of fish fed once had a similar trend to the survival of fish fed twice despite the increased feeding level. A lower growth rate in seahorses cultured in T8 was recorded compared to fish in T16. As previously mentioned, literature studies regarding seahorse culture have reported low feeding and locomotor activity during the dark period (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006), which could explain the results of T8. Seahorses probably did not feed during the 16 h scotophase gaining little or no benefit from the additional meal.

There are numerous reports on the benefits of a continuous light regime for marine fish (SilvaGarcia, 1996; Jonassen *et al.*, 2000; Simensen *et al.*, 2000; Trippel, Neil, 2003; Biswas *et al.*, 2005; 2006). However, exposure to the 24 h light did not improve seahorse growth despite the additional meal and continuous feeding opportunity. There is a possibility that the juveniles in T24 remained active, spending more energy searching for *Artemia* (which possibly also increased its activity levels becoming more difficult to catch) than the energy they assimilated by actually feeding. Coincidently, Fielder *et al.* (2002) found that a light phase longer than the natural photoperiod provided a longer feeding duration for snapper *Pagrus auratus* larvae but also extended the duration of the foraging (searching) behaviour resulting in lower weight gain of larvae cultured at 24:00 (L:D) compared to 18:06 (L:D). Similarly, Gines *et al.* (2004) reported a higher daily growth of juvenile gilthead sea bream *Sparus aurata* under an extended photoperiod of 16:08 (L:D) compared to fish cultured in tanks with continuous lighting, suggesting a greater expenditure of non-productive energy associated with increased activity.

It is often not possible to determine if the photoperiod effect on fish growth depends on food consumption or better food utilization (Trippel and Neil, 2003). It is well documented that chronically stressed teleost fish have lower growth than unstressed animals (Tucker, 1998). Diverse sources of stress in marine fish culture, such as a negative social interaction (Almazan-Rueda *et al.*, 2005), leading to lower growth and survival caused primarily by cannibalism, could be triggered by continuous light. Seahorses do not display cannibalistic tendencies; however excessive tail grasping has been reported as a cause of stress that leads to mortality as juveniles wrestle against each other instead of feeding (Woods, 2000). However, in this study such behaviour was not observed during the experiments. Stefansson

et al. (2002) suggested that the long-term culture under an extended photoperiod acted as an irritant inducing stress in juvenile turbot *Scophthalmus maximus*, suppressing growth and reducing feed utilization compared to juveniles cultured under natural photoperiod. The author attributed those findings to the development of negative size-dependent hierarchies in experimental fish under continuous light. In the present study there were no differences in seahorse size heterogeneity at the end of both experiments, which means that it was unlikely larger seahorses affected the growth of smaller juveniles in tanks under continuous light.

While no studies have directly examined feeding hierarchies in seahorses, hierarchies would appear unlikely based on seahorses' general behaviour and dispersed prey availability. It is more likely that continuous light caused a low level stress through the disruption of physiological processes and rhythmicity. In common with other chapters, most mortalities occurred in the first two weeks.

Seahorses cultured under 16:08 (L:D) exhibited increased growth when food was not limiting. Seahorses do not display conventional larval development like many other marine teleosts; therefore comparisons with photoperiod studies in other species are difficult. Some studies related to the effect of photoperiods on juvenile fish have been conducted over periods of time ranging from 60 days (Ergun *et al.*, 2003) to 12 months (Gines *et al.*, 2004) in order to observe differences among the photoperiods tested. In contrast, some studies on fish larvae have been conducted for shorter periods of time ranging between 20 to 30 days (Barlow *et al.*, 1995; Fielder *et al.*, 2002) as younger fish have an inherently faster growth than older fish (Simensen, *et al.*, 2000). In the present study, after 42 days the author was able to collect valuable data from both trials. However, future research could determine the effects of the tested photoperiods over a longer period of time.

Although the growth of early juveniles improved when cultured under an extended photoperiod of 16:08 (L:D), in the presence of a high feeding ration supplied in more than one meal, further research is required to investigate the optimal combination of photoperiod, feeding rate and meal frequency to be used in commercial seahorse production. Further analyses such as the metabolic response of the juveniles may determine nutritional stress in relation to the C: N ratio as its measurement has been used as an indicator of nutritional

stress especially in early fish stages, when the small size of the juveniles do not meet the minimum quantity of tissue required to conduct conventional proximal analyses (Harris *et al.*, 1986). The existence of melatonin profiles in seahorses could be related to their activity and physiological patterns, as with most vertebrates including several teleost species (Bromage *et al.*, 2001). Circulating levels of melatonin in the body are raised during the night and fall to basal during the day, and vary in their production patterns among species (Reiter, 1989). Based on the knowledge of these profiles, aquaculture practices such as growth and reproduction have been improved for several commercial species as melatonin has been identified in teleost as a signal to control the timing of daily and seasonal events (Randall *et al.*, 1995) and the rhythmicity of physiological processes. The small size of the seahorses at the end of the photoperiod experiments made it impossible to collect the minimum quantities of blood/plasma required to conduct radioimmunoassay (RIA) analysis for melatonin. Instead a systematic approach to the plasma melatonin production was designed for adult seahorses. The results are presented in the following chapter.

6.6 Acknowledgments

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish. Thanks to Ms Renee Florent, for her kind assistance on the improvement of earlier manuscript. Also the author would like to thank Ms Maite Lijalad for her kind assistance during seahorse rearing. The author was financially supported by Consejo Nacional de Ciencia y Tecnologia, Mexico with the additional support of Universidad Marista de Merida. Mexico.

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CHAPTER 7

LIGHT-DARK VARIATIONS IN PLASMA MELATONIN CONCENTRATIONS IN THE POT-BELLIED SEAHORSE (*HIPPOCAMPUS ABDOMINALIS*)

7 LIGHT-DARK VARIATIONS IN PLASMA MELATONIN CONCENTRATION IN POT-BELLIED SEAHORSE (*Hippocampus abdominalis*)

7.1 Abstract

Research in Chapter Six on the response of early seahorse to different photoperiods motivated the study of melatonin in this species as this hormone has several functions, the main one being the control of circadian and seasonal rhythms. Plasma melatonin was measured in adult pot-bellied seahorses *Hippocampus abdominalis* as the early juveniles cultured under different photoperiods in Chapter Six did not meet the minimum volume of blood required for analysis. The results showed that, as is common in other teleosts, the pot-bellied seahorse produces elevated levels of plasma melatonin during the scotophase, returning to basal levels during the photophase. The seahorses displayed an unusual diel melatonin profile, which described a mirror image of a type "A" profile. These results suggest that, melatonin production of the pot-bellied seahorse could be associated with the time organization of daily and seasonal events influenced by changes in photoperiod.

Keywords: *recirculation system; adult seahorse; melatonin production; validation analysis*

7.2 Introduction

The influence of photoperiod on growth and reproduction in *Hippocampus abdominalis* is poorly understood. At present, the only record of photoperiod manipulation in this species is a preliminary investigation on breeding conducted by Woods (2000) in which courtship behaviour was favoured by the extension of the photophase. In the previous chapter on photoperiod the results suggested that the decline in growth of seahorses exposed to continuous light may have been caused by the disruption of the rhythmicity of physiological processes of early juveniles as 24 h light extended the opportunity to feed. Melatonin has been associated with the endogenous rhythmicity of many biological systems through the transduction of the daily and seasonal photoperiod signal (Porter *et al.*, 2000). Therefore, the determination of plasma melatonin production patterns could contribute to a better understanding of the light-dark cycle response in seahorses. Based on this knowledge seahorse reproductive response and husbandry practices (e.g. feeding time/frequency) could be improved through photoperiod manipulation as has been achieved with another teleosts such as Atlantic salmon *Salmo salar* (Porter *et al.*, 1999).

The pineal organ processes changes in the light releasing hormonal signals such as melatonin (Pavlidis *et al.*, 1999) which influences feeding and locomotor rhythms in fish (Pinillos *et al.*, 2001). In teleosts, locomotor and physiological activities are controlled by different types of biological synchronizers which can be triggered by endogenous or exogenous cues with the light-dark cycle being one of the most common synchronizers (Vera, *et al.*, 2006). Melatonin also plays a role in the transduction of light information to reproductive and growth processes in fish (Bayarri *et al.*, 2004). Reiter (1989) defined the three main melatonin synthesis profiles: type A which presents a low melatonin level during the light phase and an increase towards the end of the scotophase; type B which maintains low levels under the light period and the production of melatonin increases to reach the maximum point in the middle of the dark phase; type C characterised by a prolonged peak during most of the scotophase. The third type is the most frequently seen melatonin production pattern in fish. The most common method to determine plasma melatonin concentrations in teleosts is the radioimmunoassay (RIA). It has been used on species such as Atlantic salmon *Salmo salar* (Randall *et al.*, 1995), Atlantic cod *Gadus morhua* (Porter *et al.*, 2000), rainbow trout

Oncorhynchus mykiss (Guerrero-Tortolero *et al.*, 2003; Larson *et al.*, 2004), river lamprey *Lampetra fluviatilis* (Mayer *et al.*, 1998), European sea bass *Dicentrarchus labrax* (Iigo *et al.*, 1997), common dentex *Dentex dentex* (Pavlidis *et al.*, 1999), tench *Tinca tinca* (Vera *et al.*, 2005), goldfish *Carassius auratus* (Iigo and Aida, 1995), swordtail *Xiphophorus helleri* (Rajchard *et al.*, 2000a), guppy *Poecilia reticulata* (Rajchard *et al.*, 2000b) and carp *Cyprinus carpio* (Kezuka *et al.*, 1988). The determination of melatonin production patterns in commercial fish species such as Atlantic salmon *S. salar* has in turn led to improvement in their commercial culture. The salmon industry experienced a production problem in the past as fish were sexually mature before they reached a harvestable size. To overcome this problem fish were exposed to continuous light to remove a daylength cue thus preventing maturation. That response is thought to be a consequence of the reduction in dark phase melatonin levels (Porter *et al.*, 1999). A similar response was found in the Atlantic haddock (*Melanogrammus aeglefinus*) as melatonin levels were suppressed in fish cultured in continuous light (Davie *et al.*, 2007). However, the response of melatonin production in fish exposed to different photoperiods can be species-specific. The same technique (12:12 L:D) used for Atlantic salmon *S. salar* in the study conducted by Porter *et al.* (1999) did not reduce the dark phase plasma melatonin when applied to Atlantic cod (*G. morua* L.) (Porter *et al.*, 2000). The author attributed the results of that study to the lees sensitive response to light of this species.

It is known that seahorses display a clear diurnal activity pattern (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006) and respond reproductively to photoperiods greater than 11 h (Woods, 2000). However, no studies on melatonin production in seahorses have been reported. The results of the previous chapter (Chapter Six) indicated that seahorses can grow at different rates relative to day length; this suggesting an influence of photoperiod on physiological processes. Therefore, the primary aim of this study is to determine the existence of melatonin in the pot bellied seahorse. The aims of this study were: 1) to conduct a radioimmunoassay of seahorse plasma to demonstrate confidently that the technique was measuring melatonin; 2) to take samples from the mid-dark and mid-light phases to determine if a difference exists in melatonin levels between the two phases; and 3) to compile a diel profile of melatonin concentrations in the blood. This information will identify if the melatonin concentration in seahorse plasma changes in relation to the

photoperiod and if so the information will form a baseline for future work on the biological rhythmicity of *H. abdominalis*.

7.3 Materials and methods

7.3.1 Animals and husbandry

Adult seahorses *H. abdominalis* of mixed sex were maintained from birth in the marine hatchery at the School of Aquaculture, University of Tasmania, Launceston. The fish were maintained in a 1 m^3 tank that formed part of a recirculating system. Seawater in the system was at a constant 32 ppt (g l^{-1}) and 17 °C. A 12:12 (L:D) photoperiod (lights on at 09:00 h, lights off at 21:00 h) was provided by a timer controlled cool white light 35 W (General Electric Company) producing an intensity of 4.8 µE s^{-1} m^{-2} at the water surface. Seahorse length (distance between the tip of the coronet to the tip of the uncurled tail) was measured by placing the fish on a submerged plastic-covered 1 mm scaled sheet. Wet weights of juveniles were measured on an electronic balance and recorded to the nearest 0.1 g. Seahorses were fed daily with *Artemia* nauplii (enriched with Super Selco® for 24 h at 17 °C) during the first four months after birth, and then on frozen mysids.

7.3.2 Blood sampling

The adult seahorses were anaesthetized by immersion in a solution of benzocaine (7.5 mg l^{-1}) in seawater. As soon as the fish lost equilibrium, they were removed and their length and weight were recorded. Blood samples were taken (100-150 µl seahorse^{-1}) via the caudal aorta using 1 ml heparinized syringes and then pooled to give approximately 600 µl aliquots. Fish were not re-sampled within each experiment. Blood was separated by centrifugation at 3000 rpm for 10 min at 4 °C and the supernatant was collected and pipetted into 1.5 ml Eppendorf tubes prior to storage at –20 °C until analysis. During the dark phase sampling under a dim red light was used to assist the operator but to avoid light-disturbance of the fish. The head of the seahorses were also covered by a damp cloth during blood withdrawal to minimise exposure to any light. There were a limited number of adult seahorses in the marine hatchery at the School of Aquaculture (University of Tasmania, Launceston). Therefore, statistical

analysis was not conducted in sections 7.3.4 and 7.3.5, as the number of aliquots obtained did not meet replication requirements. Further detail of individual sizes and sampling protocols will be provided in each section.

7.3.3 Melatonin validation analysis

All plasma samples were analysed for melatonin by direct radioimmunoassay (RIA) as described in Randall *et al.* (1995). The assay utilized sheep melatonin antiserum, (Batch 60, Stockgrand Ltd., Guilford, Surrey, UK) and [O-methyl-3H] melatonin, sp act 70-85 Ci/mmol (Amersham International Ltd., Amersham, Bucks, UK). In order to determine the presence or absence of melatonin concentrations in plasma of pot-bellied seahorses, an inhibition curve was obtained from a serial dilution (1:2) of pooled seahorse plasma collected from six adult seahorses (mean ± 1 S.E. length 18.7 ± 0.3 cm and wet weight 21.4 ± 1.4 g) at mid-dark (03:00 h) as elevated levels of plasma melatonin are produced mostly during the scotophase (Reiter, 1989).

7.3.4 Dark/light concentrations

As the elevated levels of plasma melatonin produced during the scotophase return to basal levels during day light hours in most teleosts (Reiter, 1989), additional analyses were conducted to compare the plasma melatonin levels in six seahorses sampled in the middle of the dark phase (03:00 h) to melatonin levels in six seahorses sampled at the middle of the light phase (15:00 h). Samples were obtained from adult seahorses (mean ± 1 S.E. length 17.6 ± 0.6 cm, and wet weight 16.1 ± 1.4 g).

7.3.5 Melatonin 24 h profile

To describe a diel melatonin profile in seahorses, blood samples were collected in a 24 h period divided in eight duplicated sample points (following the protocol previously described in section 7.3.2 over 24 h at: S1 (17:00-18:00 h), S2 (20:00-21:00 h -prior lights off), S3 (23:00-00:00 h), S4 (02:00-03:00 h), S5 (06:00-07:00 h), S6 (08:00-09:00 h -prior lights on),

S7 (11:00-12:00 h) and S8 (14:00-15:00 h). During each sample point two blood samples were taken for analysis. Each sample comprised blood from 4-5 seahorses. It took approximately 30 min to take one sample (Table 7.3-1). Approximately 100-250 µl of blood was extracted from each seahorse.

Table 7.3-1 Length and wet weight of adult *H. abdominalis* utilized in the analysis of plasma melatonin over a 24 h period. Sampling of each duplicate took approximately 30 minutes.

	S1 (17:00-08:00)		S2 (20:00-21:00)		S3 (23:00-00:00)		S4 (02:00-03:00)	
Sample duplicate	S1A	S1B	S2A	S2B	S3A	S3B	S4A	S4B
Number of fish sampled	5	5	5	5	5	5	5	4
Length (mm)	149.0±0.4	168.0±0.5	150.0±0.5	165.0±0.5	158.0±0.4	155.0±0.7	158.0±0.6	172.5±0.7
Wet weight (g)	8.4±1.0	12.9±0.9	9.6±0.7	11.4±1.0	11.7±1.1	10.5±1.5	11.0±0.9	15.5±1.2

	S5 (06:00-07:00)		S6 (08:00-09:00)		S7 (11:00-12:00)		S8 (14:00-15:00)	
Sample duplicate	S5A	S5B	S6A	S6B	S7A	S7B	S8A	S8B
Number of fish sampled	5	4	6	6	6	6	4	3
Length (mm)	156.0±0.5	155.0±0.8	134.1±0.3	136.6±0.3	142.5±0.7	142.0±0.3	152.5±1.1	191.6±0.1
Wet weight (g)	10.4±1.3	9.1±1.8	6.0±0.4	4.8±0.40	6.4.0±0.8	6.0±0.7	14.8±4.1	18.3±2.8

7.4 Results

7.4.1 Melatonin validation analysis

The curve showed good parallelism with the standard curve (3.9-500 pg tube^{-1}) of the assay, which was transformed from a sigmoidal curve to a linear line and the slope compared with the serial dilution of the seahorse plasma. No statistical difference in the slopes was found: $F_{1,6} < 0.001$, $P = 0.997$ using a significance level of $P < 0.05$ (ANCOVA, SPSS 11.5) (Figure 7.4-1). Assumptions of normality and homogeneity were satisfied as determined by Levene's Test and residual plots.

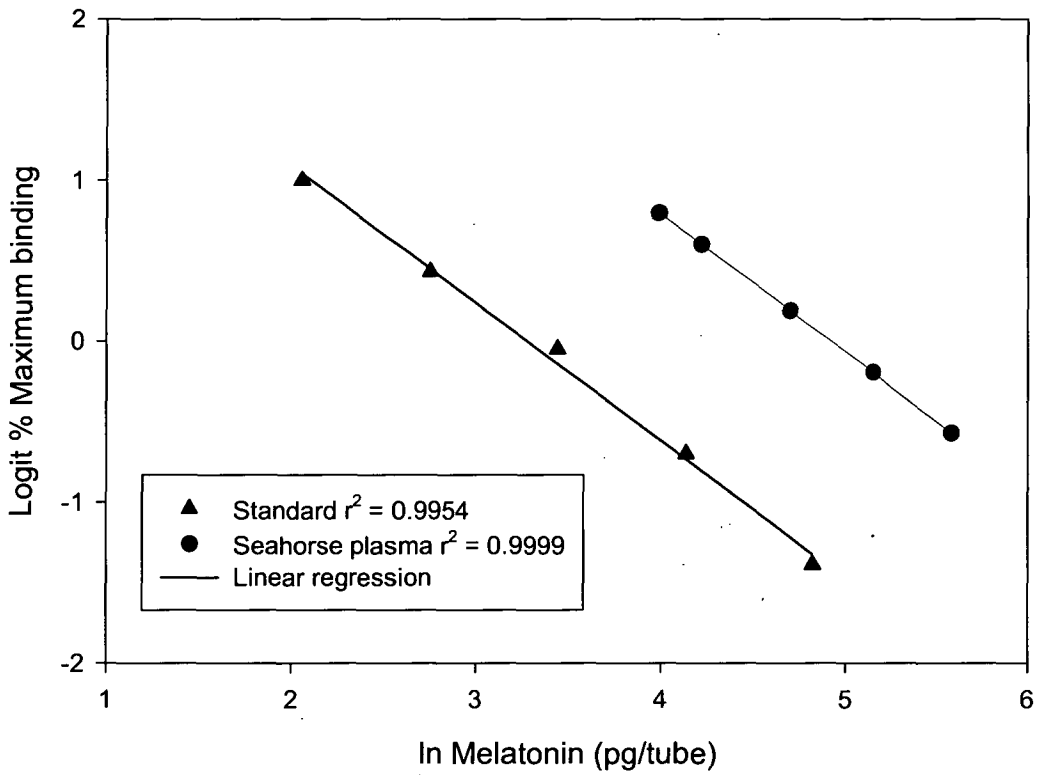

Figure 7.4-1 Parallelism of the melatonin standard curve and an inhibition curve obtained from a serial dilution (1:2) of pooled seahorse plasma (collected during the scotophase). Both curves present the logit-transformed data of the original data set. Each point represents the mean values of duplicate measurements. The X-axis denotes the natural log of the melatonin content in the standards.

7.4.2 Melatonin concentrations at mid-dark and mid-light

Day-night variations in the plasma concentrations of melatonin are presented in Figure 7.4-2. The samples taken at mid-dark showed a higher concentration of melatonin (280 pg ml^{-1}) than the samples from mid-light (168 pg ml^{-1}).

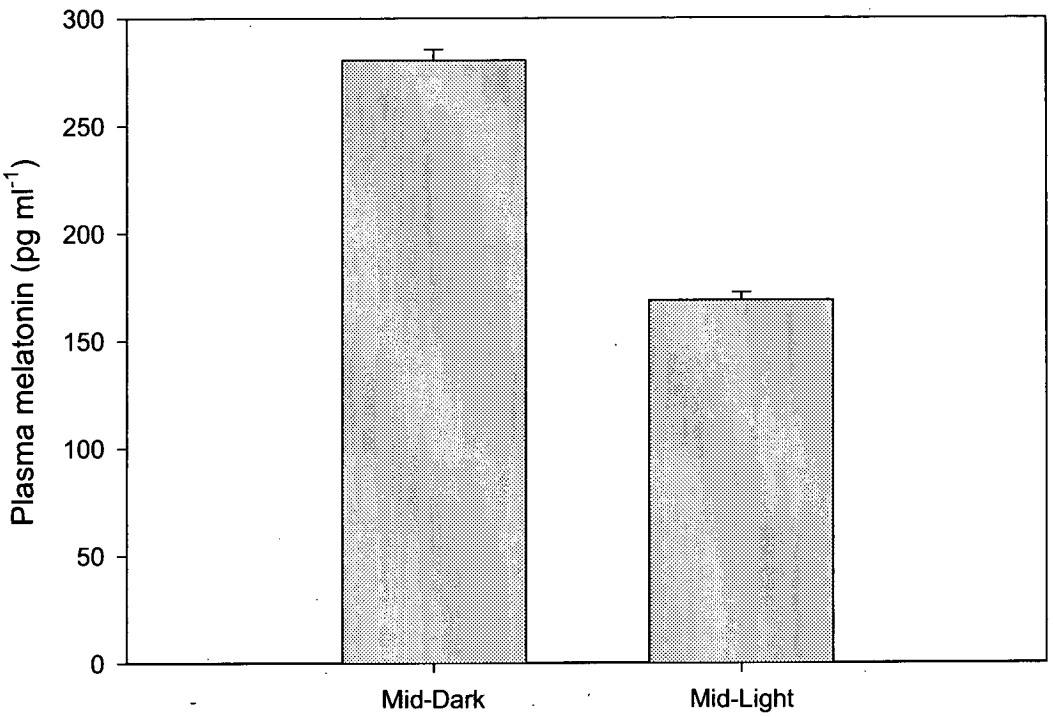

Figure 7.4-2 Plasma melatonin levels (mean ± S.E. of two replicates per treatment) at the middle of the dark period and the middle of the light period in seahorse *H. abdominalis* cultured under a 12:12 (L:D) photoperiod at a light intensity of 4.8 $\mu E\ s^{-1}\ m^{-2}$.

7.4.3 Melatonin 24 h profile

H. abdominalis exhibited a clear diel melatonin profile (Figure 7.4-3) with higher plasma levels during the dark phase reaching a maximum of 471 pg ml^{-1} at 23:00 -00:00 h, two hours after the onset of darkness. Melatonin levels decreased in subsequent samples, reaching their lowest level just before the onset of the light phase (08:00-09:00 h).

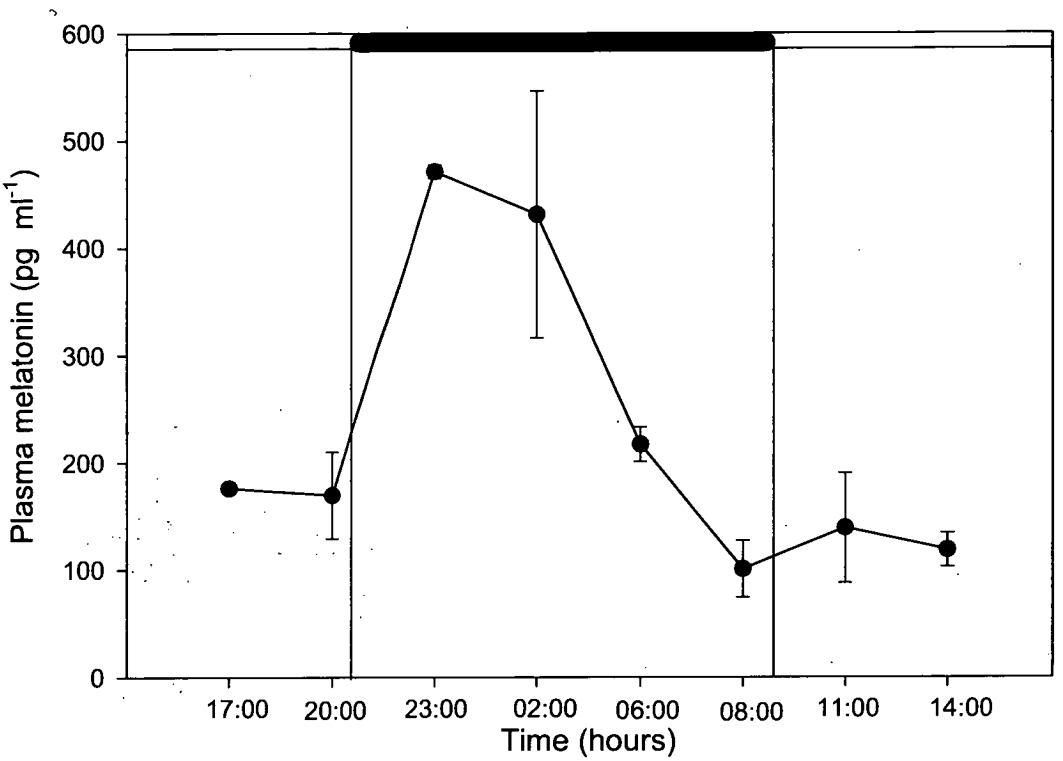

Figure 7.4-3 Plasma melatonin levels (mean of two sample-duplicates ± 1 S.E.) in seahorses *H. abdominalis* cultured under a 12:12 (L:D) photoperiod at 4.8 µE s^{-1} m^{-2}. Each duplicate sample comprised blood from 4-5 seahorses. Error bars not shown were too small to be depicted. The scotophase is represented with the black bar at the top of the graph.

7.5 Discussion

The present study demonstrates for the first time in any seahorse species that plasma melatonin is present in seahorses and furthermore that *H. abdominalis* exhibits a diel profile of melatonin over a 12:12 (L:D) period. From the three main melatonin production patterns described by Reiter (1989), the present study has recorded a production profile in seahorses similar to type B. In type B the highest level of melatonin occurs at the middle of the scotophase followed by a gradual decrease until the photoperiod starts again. However, in the present study the highest concentration of plasma melatonin was registered at the beginning of the scotophase, instead of the middle. This is unusual, and the trend was the mirror image of that in type A, in which the highest concentration of plasma melatonin is registered towards the end of the phase. Mayer *et al.*, (1998) and Kezuka *et al.*, (1988) have reported a similar plasma melatonin profile for river lamprey and common carp. Such results could suggest the existence of a fourth type of profile. However, some caution about the present results is needed because of the variability in the sample data taken at 02:00 h possibly caused by the small sample size. Unfortunately during the sampling in the present study the number of animals required to overcome this problem was not available.

Research on plasma melatonin production in fish has been conducted in juveniles and adults from which the volume of blood per fish available for sampling allows better replication compared to the volumes of blood per fish available for sampling in this study. The body morphology of seahorses is different to other commercially cultured fish species that have been analysed for melatonin production. For instance, in the study conducted by Davie *et al.*, (2007) on Atlantic haddock (*Melanogrammus aeglefinus*) the juveniles had a mean weight of 68.8 ± 0.5 g and a mean length of 167.0 ± 0.4 mm at the start of the experiment and reached almost one kilogram and more than 300 mm at the end of the experiment. The seahorses used in the melatonin profile ranged from late juveniles to fully grown adults (mean ± 1S.E. 15.5 ± 0.4 mm; 10.4 ± 0.9 g) from which it was possible to withdraw on average ± 1S.E. 156 ± 11µl of blood per animal. Therefore, it was necessary to bleed at least three seahorses to obtain the minimum amount of blood for a sample of 600 µl in order to retrieve after centrifugation the 300 µl of plasma required to conduct the RIA. As blood volume for sampling in this study was limiting even in adult fish a study of the blood

melatonin concentrations in juvenile fish held under different photoperiods as reported in Chapter Six would not be feasible. Consequently, the use of alternative melatonin sampling techniques may be explored in further research on melatonin production in seahorses. In the studies on melatonin production conducted on swordtail *Xiphophorus helleri* (Rajchard *et al.*, 2000a) and guppy *Poecilia reticulata* (Rajchard *et al.*, 2000b) the authors collected tissue (i.e. brain and eyes) instead of blood given the size of the fish used in those studies. Also the use of indirect techniques such as the water based measurement of melatonin proposed by James *et al.* (2004) could be an option in the determination of melatonin production in small seahorses.

The results of the previous chapter on photoperiod (Chapter Six) indicate that continuous light did not benefit the growth or survival of early juvenile *H. abdominalis* suggesting the influence of 24 h light on the disruption of the rhythmicity of physiological processes. Such a disruption of the melatonin production in fish exposed to photoperiods longer than the natural photoperiod has been reported for European sea bass (*Dicentrarchus labrax*) in a study conducted by Bayarri *et al.* (2004). The disruption of the diel organization of seahorses cultured in continuous light probably caused stress to some extent and suppressed growth in those fish despite the extended feeding period.

The lack of improvement in the growth of seahorses cultured under a 08:16 (L:D) (Chapter Six) suggests that despite the additional meal offered to the seahorses in 08:16 (L:D) at the onset of the scotophase, the *Artemia* were not consumed due to low feeding and locomotor activity during the dark phase as previously reported (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006). Melatonin concentrations are related to the light-dark cycle which affects food intake usually because fish are visual feeders. Possibly, the food intake was inhibited in fish exposed to a 08:16 (L:D) during the scotophase as seahorses are visual predators (Lovett, 1969).

The present study provides the first results for the better understanding of melatonin profiles in *H. abdominalis*. Further research could focus on seasonality mechanisms of melatonin action and its effects on reproduction and growth. The information provided by the previous chapter on photoperiod and the results of the present chapter suggest that seahorses could

benefit from the use of artificial photoperiod as shown with other aquacultures species. However, there is a need to examine the effect of different photoperiods on seahorse melatonin production and the link to feeding patterns/reproductive patterns in order to find optimal husbandry practices such as an appropriate feeding frequency for specific stages or appropriate L:D cycles for breeding. This will require experimentation with seahorses of a large enough size to obtain the minimal quantities of blood/plasma for the RIA or the examination of alternative body sample techniques for the determination of melatonin production patterns on small seahorses.

7.6 Acknowledgments

The author thanks Dr Natalie Moltschaniwskyj for statistical support, Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish, Ms. Renee Florent for her critical review of earlier manuscript drafts. Mr. Ryan Longland and Ms. Hanna Wolcott for their assistance with RIA analysis. The author was financially supported by Consejo Nacional de Ciencia y Tecnologia, Mexico with the additional support of Universidad Marista de Merida, Mexico.

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CHAPTER 8

SUBSTRATE-ATTACHMENT PREFERENCES OF CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*HIPPOCAMPUS ABDOMINALIS*)

8 SUBSTRATE-ATTACHMENT PREFERENCES OF CULTURED EARLY JUVENILE POT-BELLIED SEAHORSES (*Hippocampus abdominalis*)

8.1 Abstract

Seahorses spend a large proportion of their life attached to a diversity of materials particularly during the dark phase or when not foraging for food. In order to determine the substrate preferences of the pot bellied seahorse *Hippocampus abdominalis*, early juveniles were provided with three choices in diameter of nylon monofilament: 0.17 mm, 0.55 mm, 0.90 mm and plastic mesh-density: 5 mm, 10 mm and 24 mm bar–length (length of each of the four equidistant segments that form one mesh square) in a series of trials. Two seahorse life-stages were observed: 28-day-old and newborns. In all trials observations of attached to substrate, swimming, or stationary on the bottom of the tank were recorded pre-feeding at 10:30 h. Juveniles were fed with live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % body weight day^{-1} (BW d^{-1}) (dry weight *Artemia*: wet weight fish), with all other observations taken at 30 min intervals following feeding. Twenty eight-day-old juveniles exhibited a consistent pattern of decreasing activity after feeding. The number of seahorses attached to the largest diameter substrate and the lowest mesh density was greater than the number of seahorses found on the smallest diameter substrate and highest mesh density. Newborns were provided with the same substrate choices as 28-day-old juveniles and two in-tank conditions: aeration and no aeration. The substrate preference of newborns exposed to aeration was not different to that of seahorses without aeration. In general, newborns preferred to hold on to larger substrates rather than small substrates, consistent with the 28-day-old juveniles. However, newborns did not show a pattern of decreasing locomotor activity after feeding as did 28-day-old juveniles.

Keywords: *recirculation system; mesh density; substrate diameter; fish behaviour*

8.2 Introduction

Seahorses display unique morphological features including a right-angled head, stretched skin over a series of cartilaginous rings and a prehensile tail (Gomon and Neira, 1998; Kuiter, 2000). The prehensile tail allows seahorses to temporarily hold onto substrates such as sponge, branching coral, seaweeds or submerged tree branches in their natural habitat, and also onto artificial structures such as aquaculture cage nets (Foster and Vincent, 2004).

Seahorses spend a large proportion of their life attached to a diversity of materials (Foster and Vincent, 2004) particularly during the dark phase (Ouyang, 2005) or when not foraging for food as observed by Karina *et al.* (2006) on cultured adult long-snout seahorse *Hippocampus reidi*. The latter authors reported the utilization of two different materials in artificial plants and calcareous artificial substrate. Wong and Benzie (2003) provided substrate to late juvenile *Hippocampus whitei* in the form of artificial seagrass (made from thin black plastic strips) and tank-bottoms covered with black plastic fencing-mesh. In general, experiments on *Hippocampus abdominalis* have made regular use of certain items such as individual strands of separated black shade cloth of 1 mm diameter (Woods, 2003a; b; c; Woods and Valentino, 2003), plastic mesh (Adams *et al.*, 2001; Shapawi and Purser, 2003; Wilson *et al.*, 2006) and artificial plants (Woods and Martin-Smith, 2004;Woods, 2005).

Under mass culture conditions, it would be desirable to use a substrate of simple form and easy to clean, hence the selection of substrates such as rigid plastic mesh by some companies (Seahorse World Pty. Ltd.). Most of the seahorse research studies have been conducted on late juveniles, with only a few experiments using plastic shade cloth on early stage juveniles from birth (Woods, 2000a; Woods, 2003a). In general, early seahorse stages are predominantly pelagic becoming benthic after 2-3 weeks (Choo and Liew, 2006); this characteristic has been taken into account while conducting experimentation in captivity. Wilson and Vincent (1998) provided no substrate to juvenile seahorses (*Hippocampus fuscus, Hippocampus barbouri* and *Hippocampus kuda*) during the first 28 days of their life; then artificial macro-algae when juveniles became more sedentary. Scarratt (1996) increased the size of the substrate provided to early juveniles *Hippocampus erectus* from monofilament

line with newborns, to artificial eelgrass (0.3 cm blade width) at day nine after birth to a width-increased 0.5 cm artificial eelgrass from day 50 after birth onwards. While researchers have utilized a range of substrate types and sizes, studies have not specifically tested the substrate preferences of seahorses, especially those of early juveniles.

H. abdominalis is considered pelagic during the first month of life (Gomon and Neira, 1998). However, in previous research conducted by the author in the present study it was noticed that early juveniles attached to the substratum provided (i.e. a weighted bundle of fishing line filaments and a floating plastic mesh). The attachment to substrates has an important role in numerous aspects of seahorse biology (Hale, 1996). In seahorse culture, the current produced by in-tank aeration can cause a positive effect as animals tend to cling to substrate removing them from exposure to the water surface which appears to increase the likelihood of swim bladder hyperinflation. Juvenile seahorses unable to hold on to substrate attachment are more susceptible to swimmbladder related problems than seahorses provided with substrate (Florent, 2003). In late stages, holding on to attachment substrate plays a role in some courtship displays (Choo and Liew, 2006). Seahorses are visual predators, as they are not high-speed swimmers; they adopt a sit and wait ambush strategy (Lovett, 1969). Therefore, seahorses attached to an appropriate substrate during an ambush may improve their predation efficiency compared to fish holding on to less suitable (e.g. less stable) substrates. Seahorses tend to be more demersal as they become older, and hence tend to attach more frequently than young juveniles (Gomon and Neira, 1998). The aim of this study was to determine if early juveniles (newborn, 28-day-old) of *H. abdominalis* exhibited any preference for a particular size or density of the substrate. The specific objectives were: 1) to test the preferences of two stages of seahorses for three substrate diameters and three substrate densities; and 2) to examine the effect of in-tank aeration compared to the lack of in-tank aeration in the preference for three substrate diameters and three substrate densities.

8.3 Materials and methods

8.3.1 System design and general

Juvenile seahorses were transported in seawater 33 ppt (g l^{-1}) at 17 °C and oxygen-filled

plastic bags inside an insulated container from a commercial seahorse farm (Seahorse World Pty. Ltd. Beauty Point) to the marine hatchery in the Aquaculture Centre at the University of Tasmania, Launceston. After a 15 min temperature acclimation the fish were allocated to a 20-l holding tank until experimentation. A ring of light-coloured plastic mesh with 3 cm bar-length (the length of each of the four equidistant segments that form one mesh square) and 3 mm bar diameter was placed in the holding tank providing attachment substrate for seahorses before experiments. Transparent 3-l tanks forming part of a 100-l recirculation system were utilised for the experiments. The recirculation system included a biofilter comprised of two stacked 40-l plastic containers. The upper container was filled with 40-mm bio balls and its floor area perforated every five centimetres to allow the outflow water from the tanks to trickle down to the container below. This lower container was used as a water reservoir in which was installed a 40 W submersible pump of a 2800 l h^{-1} delivery volume (Resun®) that provided an inflow of approximately 2.5 l hr^{-1} $tank^{-1}$of 20μm filtered seawater. A 12:12 (L D) photoperiod was provided (lights on at 08:00 h, lights off 20:00 h) by a timer controlled cool white fluorescent light 35 W (General Electric Company), providing a light intensity of 4.8 μE s^{-1} m^{-2} at the water surface. Water quality was maintained as follows: salinity (mean ± 1 S.E.) 32.7 ± 0.1 ppt, pH 7.8 (range 7.5-8.0), dissolved oxygen > 75 %, total ammonia nitrogen (TAN) < 0.5 mg l^{-1}, nitrite < 0.25 mg l^{-1} and nitrate < 5 mg l^{-1}. Seahorse length (distance between the tip of the coronet to the tip of the uncurled tail) represented fish size and was measured by placing the fish on a 1 mm scaled sheet covered by plastic. Wet-weight of seahorses was measured on an analytical balance and recorded to the nearest 0.0001g.

8.3.2 Substrate preference (28-day-old juveniles)

The aim of these short-term observations was to determine the preference of juvenile seahorses provided with the choice of various diameters of attachment substrate in different positions and densities. In-tank aeration was not provided. Juveniles (mean ± 1 S.E. length = 29.8 ± 0.1 mm, mean ± 1 S.E. wet weight = 43.4 ± 1.0 mg) from a single brood (28-day-old) were used in this series of experiments. Twenty seahorses were randomly distributed to each 3-l transparent tank and were left for 30 min prior to the first observation. Visual observations on the number of fish attached to each of the nylon filament-diameters or mesh-

densities were conducted. Swimming seahorses were included as a separate category. A pre-feed observation was recorded at 10: 30 h, immediately after the juveniles were fed with live *Artemia* (enriched with Super Selco® for 24 h at 17 °C) at a rate of 14 % body weight day^{-1} (BW d^{-1}) (dry weight *Artemia*: wet weight fish) (dry weight *Artemia*: wet weight fish). Observations were made every 30 min thereafter.

After observations seahorses from all experimental tanks were mixed and returned to a 20-l holding tank. For subsequent repetition of experiments, seahorses were randomly selected each time.

8.3.2.1 Diameter preference

Sections of transparent nylon filament of three diameters: 0.17 mm, 0.55 mm and 0.90 mm were wound on a square frame (3 mm diameter); it provided three choices of substrate attachment, all from the same material. The frames were placed horizontally in three tanks and vertically in the other three.

Observations were conducted over a 2-h period as described in section 8.3.2. The experiment was repeated the following day with different fish but maintaining the same substrate arrangement and orientation.

8.3.2.2 Mesh-density preference

A rectangular sheet of plastic mesh (bar-length of 5 mm, diameter 1 mm) was offered as an attachment substrate. The sheet was modified by removing some of the bars in order to maintain the same material and mesh diameter and provide three choices of mesh densities: 5 mm, 10 mm and 24 mm bar-length. The sheets were placed horizontally in three tanks and vertically in the other three.

Observations were conducted over a 2-h period following the same protocol described in 8.3.2. The experiment was repeated the following day, maintaining the same substrate arrangement and orientation.

8.3.2.3 Diameter preference: a frame-less approach

Sections of transparent nylon filament of three diameters: 0.17 mm, 0.55 mm and 0.90 mm were glued to a non-toxic weight. Each structure resembled a sphere providing three rings (one 30 cm ring per size) with the diameter choices of substrate attachment (Figure 8.3-1). Seahorses were randomly distributed in three 3-l tanks in this experiment instead of six 3-l tanks as in section 8.3.2.1 because orientation (horizontal/ vertical) was not a consideration with the new structural shape.

Observations were conducted over a 5-h period following the same protocol described in section 8.2.3 .The experiment was repeated over three consecutive days.

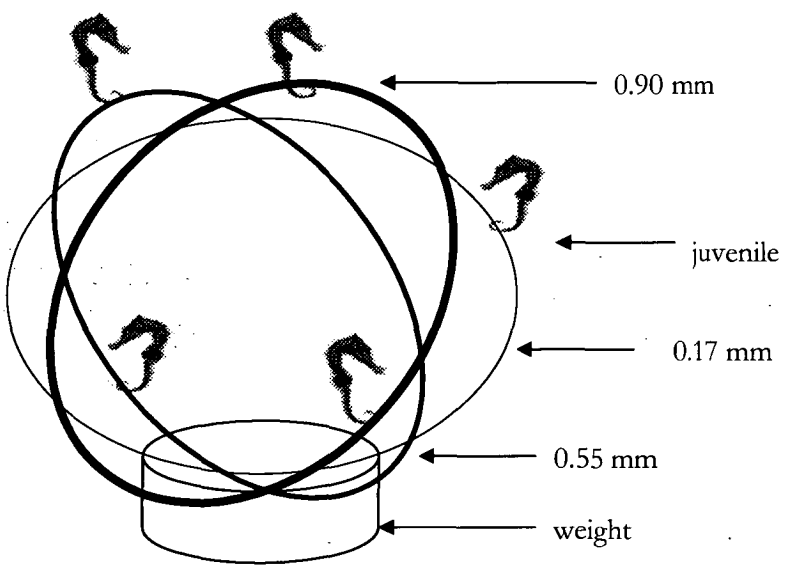

Figure 8.3-1 Frameless substrate-structure; sections of transparent nylon filament of three diameters 0.17 mm, 0.55 mm and 0.90 mm were glued to a non-toxic weight.

8.3.3 Substrate preference (newborn seahorses)

8.3.3.1 Diameter preference

The substrate-structure described in section 8.3.2.3, was used in this experiment. In order to observe the effect of continuous aeration on substrate preference three 3-l tanks were provided with aeration tubing ending with a 4-l h^{-1} plastic water-dripper (Neta®) located in the bottom of the tank and another three 3-l tanks remained without aeration. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish.

8.3.3.2 Mesh-density preference

The substrate-structure described in section 8.3.2.2 was placed horizontally in six 3-l tanks for this experiment. In order to observe the effect of continuous aeration on substrate preference three 3-l tanks were provided with aeration tubing, ending with a 4-l h^{-1} plastic water-dripper (Neta®) allocated in the bottom of the tank and another three 3-l tanks remained without aeration. Aeration was not located under the substrate but adjacent to and removed from the substrate to avoid direct disturbance to the fish. Simultaneously the same mesh structure was placed vertically in another six 3-l tanks providing aeration to only 3 tanks and the other 3 tanks remained without aeration.

8.3.4 Data presentation and analysis

The aim of the study was to determine if juvenile seahorses displayed any substrate preference and how consistent it was over time. A χ^2 independency test ($P < 0.05$) was used to determine if the proportion of seahorses on each substrate choice was significantly different across the observations taken each day.

8.4 Results

8.4.1 Substrate preference of 28-day-old juveniles

8.4.1.1 Diameter preference

Horizontal orientation

From the first observation (before feeding) the need to consider an additional substrate (frame) was evident as a considerable number of seahorses were attached to the 3 mm frame. Based on the number of seahorses counted in each substrate choice in most observations the highest frequency was recorded on the 3mm frame followed by the 0.90 mm diameter (Table 8.4-1). The remainder of substrate choices (0.55 mm and 0.17 mm) were the least frequented. This distribution was not statistically different over time ($\chi^2 = 11.974$, df = 12, $P = 0.448$). Repetition on the following day exhibited a similar distribution to that described on day one and the distribution was not statistically different over time ($\chi^2 = 15.095$, df = 12, $P = 0.236$).

On day one, at the beginning of the observations the number of fish swimming was higher than the total number of fish attached to the three substrate choices. However, this distribution changed significantly for the rest of the observations, displaying similar numbers of fish swimming compared to the total number of fish attached to the three substrate choices ($\chi^2 = 12.525$, df = 4, $P = 0.014$). On day two, the number of fish swimming was similar to the total number of fish attached to the three substrate choices and did not change significantly over time ($\chi^2 = 7.683$, df = 4, $P = 0.104$).

Vertical orientation

On day one the proportion of seahorses in each substrate choice was marginally different over time ($\chi^2 = 21.202$, df = 12, $P < 0.047$). However, the trend was somewhat consistent with the horizontal orientation; in which the 3 mm frame recorded the highest frequencies, followed by the 0.90 mm diameter (Table 8.4-1). In this experiment, the lowest frequency was recorded in the 0.17 mm diameter filament in most observations. When repeated on the following day, the trend was consistent with that on day one, except the distribution was not significantly different over time ($\chi^2 = 10.667$, df = 12, $P < 0.558$).

On day one, at the beginning of the observations the number of fish swimming was higher than the total number of fish attached to the three substrate choices. However, this distribution changed significantly for the rest of the observations, (χ^2 = 18.462, df = 4, P = 0.001). The distribution in the first observation on day two was consistent with the distribution in the first observation on day one and also changed significantly over time (χ^2 = 38.705, df = 4, $P < 0.0001$).

8.4.1.2 Mesh-density preference

Horizontal orientation

The seahorses changed their preference for a specific attachment choice over time (χ^2 = 16.543, df = 8, P = 0.035). However, in most observations the highest frequencies were recorded in the 24 mm bar-length mesh density and lowest frequencies were recorded in the 5 mm bar-length mesh density (Table 8.4-1). On the following day, despite high variability, a similar trend to that of day one was recorded in most observations (χ^2 = 26.035, df = 8, P = 0.001).

On day one, the number of fish swimming was similar compared to the total number of fish attached to the three substrate choices. This distribution was consistent over time (χ^2 =7.251, df = 4, P = 0.123) and was also consistent over time on day two (χ^2 = 6.140, df = 4, P = 0.189).

Vertical orientation

On day one seahorses predominantly attached to the 24 mm bar-length mesh as the highest frequencies were recorded in this substrate choice (Table 8.4-1), and this distribution was not statistically different over time (χ^2 = 6.204, df = 8, $P < 0.624$.). When repeated the following day, the distribution of the juveniles was similar to that of day one and was not statistically different over time (χ^2 = 10.587, df = 8, P = 0.226). On both days the lowest number of juveniles were observed holding onto the 10 mm bar-length mesh density instead of the 5 mm as in the majority of the tests (Table 8.4-1).

On day one, at the beginning of the observations the number of fish swimming was higher compared to the total number of fish attached to the three substrate choices. However, this distribution changed significantly over time ($\chi^2 = 33.307$, df = 4, $P < 0.0001$). Consistent with day one, at the beginning of observations on day two, the number of fish swimming was higher compared to the total number of fish attached to the three substrate choices and also there was significant variability over time ($\chi^2 = 14.965$, df = 4, $P = 0.005$).

Table 8.4-1 Total number of 28-day-old *H. abdominalis* recorded in each of the three substrate choices provided over two observation days. The last two rows in each day indicate the total number of seahorses recorded swimming or attached to the three substrate choices.

Substrate choice tested		Diameter	Diameter	Density	Density
Position		horizontal	vertical	horizontal	vertical
Day 1	Substrates in order of preference	(3 mm) 70	(3 mm) 61	(24 mm) 59	(24 mm) 73
		(0.90 mm) 47	(0.90 mm) 35	(10 mm) 44	(0.55 mm) 58
		(0.55 mm) 12	(0.55 mm) 25	(0.55 mm) 29	(10 mm) 25
		(0.17 mm) 6	(0.17 mm) 9		
	Swimming	165	170	168	144
	Attached	135	130	132	156
Day 2	Substrates in order of preference	(3 mm) 86	(3 mm) 73	(24 mm) 85	(24 mm) 84
		(0.90 mm) 40	(0.90 mm) 29	(10 mm) 40	(0.55 mm) 29
		(0.55 mm) 14	(0.55 mm) 21	(0.55 mm) 30	(10 mm) 14
		(0.17 mm) 7	(0.17 mm) 17		
	Swimming	153	160	145	173
	Attached	147	140	155	127

8.4.1.3 Diameter preference: a frame-less approach

On day one in most observations the majority of the attached juvenile seahorses settled on the 0.90 mm diameter substrate, which remained the most preferred until the last observation. The 0.17 mm diameter-filament recorded the lowest frequencies. This distribution was not consistent over time ($\chi^2 = 40.091$, df = 20, $P = 0.005$). The counts on replicate days showed that the seahorses displayed a preference for 0.90 mm diameter substrate, followed by the substrates 0.55mm and 0.17 mm respectively and there were no significant differences throughout the sampling period on day two ($\chi^2 = 28.557$, df = 20, $P = 0.097$) and day three ($\chi^2 = 23.071$, df = 20, $P = 0.285$).

At the beginning of the observations on day one, the number of fish swimming was similar to the number of fish attached to any substrate choice. After that the distributions changed as more seahorses became attached throughout the rest of the observations ($\chi^2 = 46.518$, df = 10, $P < 0.0001$). On day two and three, the highest number of swimming seahorses was recorded in the pre-feeding observation. After that the distribution varied in the remaining observations in the number of fish swimming compared to the total number of fish attached to the three substrate choices ($\chi^2 = 60.911$, df = 10, $P < 0.0001$) ($\chi^2 = 28.451$, df = 10, $P = 0.002$).

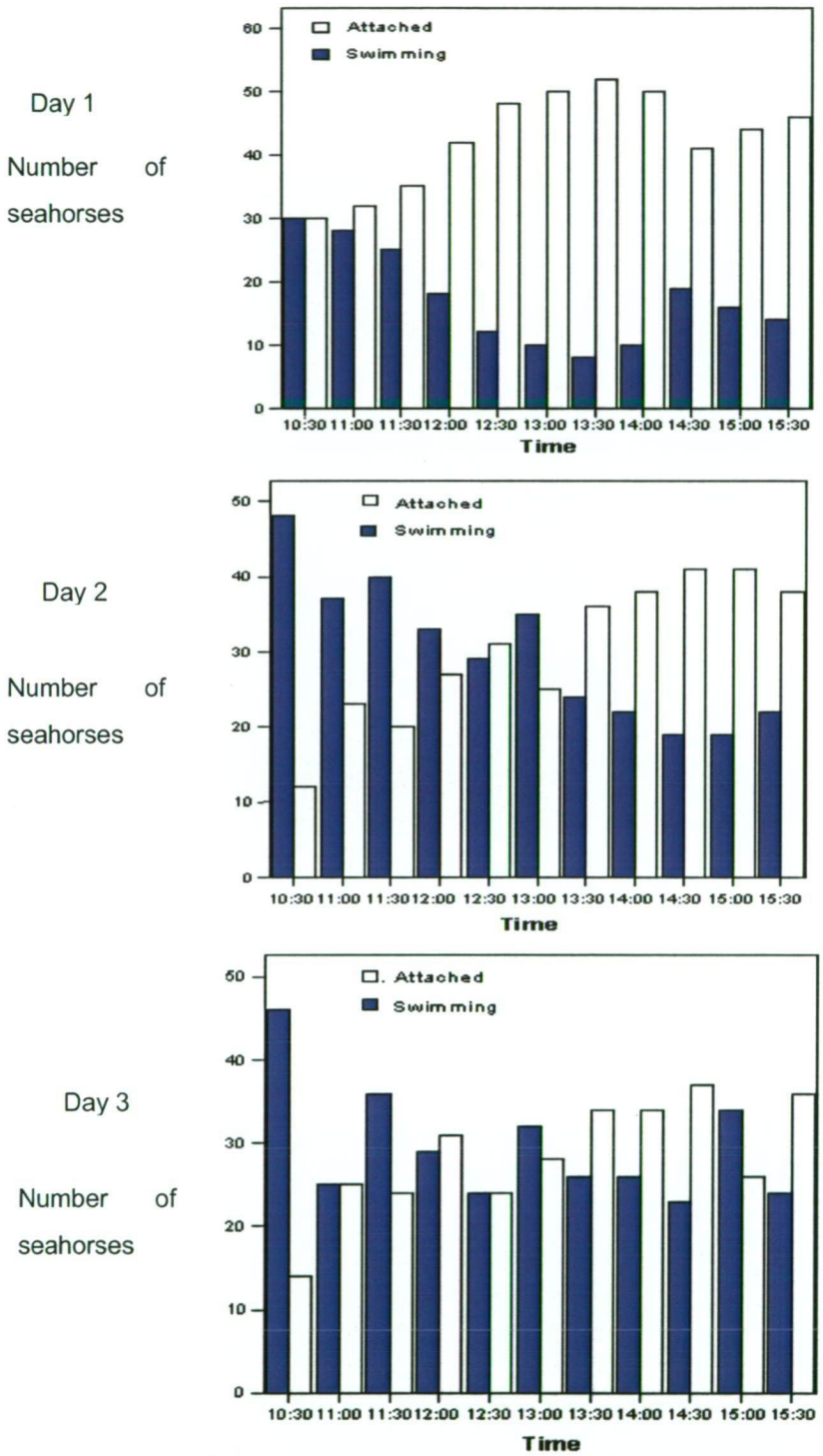

Figure 8.4-1 Number of seahorses recorded swimming compared to the total number of seahorses attached to three diameter substrate choices (0.90, 0.55 and 0.17 mm) over time in three days. The observation at 10:30 was taken before feeding, with all other observations taken at 30 min intervals.

8.4.2 Substrate preference of newborn seahorses

8.4.2.1 Diameter preference

Aeration

As with experiments conducted on 28-day-old seahorses, newborns displayed a preference for the 0.90 mm diameter substrate in most observations. However, the distribution of the seahorses in each substrate was different over time ($\chi^2 = 30.432$, df = 12, $P = 0.002$). The 0.17 mm filament showed the lowest seahorse frequencies. The number of fish swimming was similar to the total number of fish attached to the three substrate choices and this distribution was not significantly different over time ($\chi^2 = 2.915$, df = 6, $P = 0.819$).

Without aeration

Newborns displayed a preference for the 0.55 mm diameter substrate in most observations, followed by the 0.9 and 0.17 mm diameter substrates. However, the proportion of seahorses attached to each substrate choice was different over time ($\chi^2 = 34.063$, df = 12, $P = 0.001$). The 0.17 mm diameter substrate appeared to be the least preferred by newborn seahorses in this experiment. The total number of fish attached to the three substrate choices was greater than the number of fish swimming and this distribution was not significantly different over time ($\chi^2 = 3.750$, df = 6, $P = 0.710$).

8.4.2.2 Mesh-density preference

Horizontal with aeration

Seahorse substrate preference was not significantly different throughout the sampling period ($\chi^2 = 19.694$, df = 12, $P = 0.073$). In all observations the highest frequency was recorded on the 24 mm substrate choice, followed by the 10 mm and 5 mm mesh density. The total number of fish swimming was greater than the total number of fish attached to the three substrate choices in most observations. However, there was significant variability in fish distribution over time ($\chi^2 = 17.705$, df = 6, $P = 0.007$).

Horizontal without aeration

The proportion of seahorses in the 10 mm bar-length mesh-density section showed the highest frequency in most observations followed by 24 mm and 5 mm respectively. Seahorses did not remain in a specific mesh density throughout the sampling period (χ^2 = 31.873, df = 12, P = 0.001). Although the sum of the frequencies of fish swimming and the sum of the total number of fish attached to the three substrate choices were similar the distributions varied significantly over time (χ^2 = 32.689, df = 6, P < 0.001).

Vertical with aeration

Seahorse substrate preference was significantly different throughout the sampling period (χ^2 = 30.478, df = 12, P = 0.002). However, the 24 mm bar-length mesh density showed the highest frequency in most of the observations followed by 10 and 5 mm respectively. The total number of fish attached to the three substrate choices was similar to the number of fish swimming and this distribution was not significantly different over time (χ^2 = 6.457, df = 6, P = 0.374).

Vertical without aeration

The number of seahorses holding on to the 24 mm bar-length mesh density was higher than those on the 10 mm in most observations. However, seahorses did not remain on a specific substrate over time (χ^2 = 29.516, df = 12, P = 0.003). The total number of fish attached to the three substrate choices was greater than the number of fish swimming in most of the observations but there were significant changes to this distribution over time (χ^2 = 16.427, df = 6, P = 0.012).

Table 8.4-2 Total number of newborn *H. abdominalis* recorded on each of the three substrate choices provided on one observation day. The last two rows indicate the total number of seahorses recorded swimming or attached to the three substrate choices.

	Diameter aeration	Diameter without aeration	Density aeration horizontal	Density without aeration horizontal	Density aeration vertical	Density without aeration vertical
Substrates in order of preference	(0.90 mm) 117	(0.55 mm) 123	(24 mm) 117	(10 mm) 100	(24 mm) 120	(24 mm) 105
	(0.55 mm) 63	(0.90 mm) 93	(10 mm) 53	(24 mm) 77	(10 mm) 59	(10 mm) 100
	(0.17 mm) 33	(0.17 mm) 36	(5 mm) 35	(5 mm) 31	(5 mm) 30	(5 mm) 24
Swimming	207	168	215	207	211	201
Attached	213	252	205	208	209	229

8.5 Discussion

This is the first study to report on the substrate preferences of seahorses at the stage of newborns and 28-day-old. Seahorses displayed a discriminant use of substrate as they tended to hold onto large diameter (0.9 mm) and low mesh density (24 mm bar-length) substrates. The present study aimed to sample as rigorously as the experiment and circumstances would allow by providing data on the material size and the seahorse preference of different substrate diameter/density over time. The time range of observations was based on a study conducted by Ouyang (2005), who found that *H. abdominalis* displayed decreased swimming activity after feeding, as they are predominately active when hungry. This approach was important to the study, as the measurements were reliant on attached fish. Based on the results of the present study, further research is needed to test larger diameters and mesh densities of the same material, as indicated by the greater frequencies recorded in the 3 mm frame compared to the frequencies recorded in the 0.90 diameter substrate during the first trial. Despite some variability, it appeared that seahorse preference decreased proportionally with the diameter/density of the substrate as indicated by the frequencies recorded in 0.55 mm and 0.17 mm for diameter and 10 mm and 5 mm for mesh density respectively which followed the frequencies recorded in 0.9 mm diameter and 24 mm mesh density.

The incidental inclusion of the 3 mm frame as a substrate choice during the first trial motivated the design of a specific substrate structure to avoid the edge effect of a frame in a second trial. The spherical frameless structure used in the second trial, confirmed the 28-day-old seahorse's preference for the largest substrate choice which was the 0.90 mm diameter substrate. This tendency was consistent with the trial on newborns. However, there was more variability on the seahorse's substrate preferences as indicated by the preference for the 10 mm mesh density and the 0.55 mm diameter (frameless structure) instead of the 24 mm mesh density and 0.90 mm diameter respectively in horizontal position in the absence of aeration.

In the present study, the number of seahorses swimming during observations was not included in the comparisons among substrate choices. Instead, the number of seahorses swimming was compared to the total number of seahorses attached to the three substrate

choices. In the trials with 28-day-old seahorses it was often noticed that in the first observation (pre-fed) the number of seahorses swimming was greater than the number of seahorses attached this was more evident in the second trial on day two and three. On those days, seahorses appeared to use the substrate after feeding which possibly affects the accuracy of the feeding strikes. These results are consistent with those of Ouyang (2005) who reported juvenile *H. abdominalis* actively foraging when hungry (pre-fed) and sedentary when satiated. This post-prandial behaviour was less evident in newborns.

In a separate preliminary observation on 2-day-old juveniles, the fish allocated in experimental tanks were observed to rise to the surface, a behaviour previously reported for this species (Gomon and Neira, 1998; Lovett, 1969). Such behaviour resulted in the entire brood developing an acute swim bladder hyperinflation problem; hence no data on substrate preference was obtained. Juveniles concentrated at the surface held onto each other and were unable to feed effectively. Woods (2000b) reported that in the absence of substratum, "balls" of juveniles can occur resulting in stress or even death as juveniles wrestle against each other and were prevented from feeding. In seahorse culture, the use of a continuous aeration appears to improve survival by preventing or reducing swimbladder hyperinflation in early juveniles (Florent, 2003). However, in the present study, the aeration provided to juveniles did not appear to have a significant effect on swimbladder hyperinflation compared to those without aeration. The use of aeration did not appear to affect differently either the substrate preference or the tank distribution (swimming/ attached) of juveniles, compared to seahorses in tanks without aeration. The aeration provided in this study was located in the bottom of the tank to provide similar conditions to those used in commercial seahorse culture (Seahorse World Pty. Ltd. pers. comm.).

The number of fish used in this study was selected based on different aspects such as the stocking density used in commercial seahorse culture and statistical requirements, for which a minimum of five per substrate choice was required in each tank. While the behaviours of fish are not necessarily independent of one another (i.e. one fish attaching may stimulate others to follow) the results measured the response of a group of fish to the presence of substrate. This was not pseudoreplication because the fish were not the replicates but rather the tank response is the replicate. Further research is needed to examine the effect that

seahorse interaction could have on substrate preference. While no studies have directly examined feeding hierarchies in seahorses, hierarchies would appear unlikely based on the seahorse's general behaviour and dispersed prey availability in this study.

Woods (2000b) reported in a preliminary breeding-protocol that for the first 14 days after birth, juveniles congregated near to the water surface, either free-swimming, or attached to shade cloth strands (1 mm diameter). Once they reached 25 mm in length and 0.4 g in wet weight juveniles started to move away from the surface, spending less time swimming and more time holding onto the available substratum. The results of the present study are contrary to the finding of Woods (2000b). Although there was more variability on the preference of the newborn seahorses, more than a half of the newborns observed where attached to substrate and tended to prefer large rather than small substrates. Perhaps, in the present study seahorse preference for large substrates was caused by pre-conditioning, since seahorses were previously exposed to 1 mm diameter and 3 cm bar-length for four weeks in the case of the 28-day-old fish. However, a one-day pre-conditioning in the newborns used would appear unlikely.

Scarrat (1996) recommended for *H. erectus* rearing, the use of monofilament for newborns (no specified size) and larger diameter substrates (compared to those used in the present study) after nine days after birth in the form of artificial eelgrass of 0.3 cm blade width, switching on day 50 after birth to artificial eelgrass of 0.5 cm width. However, that recommendation was made on the assumption that small seahorses would prefer small substrates but there were no comparisons conducted. The present study took a systematic approach as it used a specific diameter range (0.17, 0.55 and 0.90 mm) of an accessible, visually-homogeneous and accurately sized material of nylon monofilament. The present study considered the inclusion of a reference size (0.90 mm) close to that (1 mm) used previously in early *H. abdominalis* experimentation (Woods, 2000a; Woods, 2003a).

This study was conducted under a multiple-choice design in which seahorses were provided with three different diameter/densities from which they appeared to prefer the largest choice. Future research can aim to determine the adaptability of fish by examining their response when given a less preferred substrate (i. e. smaller) as the only choice. Further work could

also examine the interaction between seahorse stocking density and substrate density, relative to patterns of swimming and attachment. The seahorse industry requires more research on cost-effective husbandry solutions to seahorse requirements. The substrate preference of the pot-bellied seahorse is an important issue in order to provide an optimal distribution of fish within culture tanks. Based on the results of this study the use of substrate diameters not smaller than 1 mm, and mesh densities of 24 mm in bar-length is recommended.

8.6 Acknowledgments

The author thanks Dr Natalie Moltschaniwskyj for statistical support and Seahorse World Pty. Ltd. Beauty Point, for providing the experimental fish. Thanks to Ms Renee Florent, for her kind assistance on the improvement of earlier manuscript drafts. The author was supported by CONACyT Mexico with the additional support of Universidad Marista de Merida, Mexico.

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CHAPTER 9

GENERAL DISCUSSION

9 GENERAL DISCUSSION

This study was undertaken to answer a number of questions on seahorse husbandry posed by research and commercial ventures at that time. While seahorse culture of early juveniles was successful, many husbandry protocols were developed in an *ad hoc* and trial/error approach rather than by systematic and replicated methods which formed the strategy in this study.

This study considered the sequential testing of six environmental factors (tank colour, temperature, stocking density, salinity, and photoperiod) involved in seahorse culture presented as chapters, in which all possible care was taken to maintain the consistency of the protocols. In addition, findings from some chapters were incorporated into the methods of other chapters. For instance, Chapter Seven examined a physiological issue in the determination of melatonin, from a question generated in Chapter Six on photoperiod. As a logical culmination of this project, the optimal levels found in each section should be tested altogether; unfortunately time restrictions, resources and fish availability made this unfeasible. Instead, the following discussions will examine common issues of the different chapters and the possible combinations of the factors tested individually which can constitute the basis of future research protocols.

In commercial fish culture there is an interaction of environmental factors, which ideally should be tested simultaneously within a multi-factorial matrix, requiring large numbers of juveniles that were unavailable at the time this study was conducted. Seahorses give birth to relative small broods compared to pelagic-egg species (e. g. flounder). Therefore, large quantities of fish of the same age are not often available for seahorse research. However, thanks to the participation of Seahorse World Pty. Ltd. the author had access to a reasonable number of early juvenile seahorses in a regular supply throughout the year to satisfy experimentation and replication requirements, making this study possible.

The present study examined the effects of some of the most important environmental factors in early seahorse culture and was motivated by the lack of scientific information in these early stages. The ranges used in this study were selected from the ranges used in commercial

seahorse culture and the ranges that *H. abdominalis* experiences in its natural environment. The range of colours tested in Chapter Two did not produce significant differences in growth or survival. However, it was noticed that during mortality inspections and tank cleaning, the light coloured tanks provided a better contrast allowing an easier observation of the number and condition of the fish which ultimately can improve commercial culture practices. The lack of significant differences was due in part to the variability of data, which may have been overcome by more extensive sampling. Further research could test less colour choices (instead of six) with more replicates. While conducting further experiments, the use of an adjusted feeding ration to avoid possible confounding effects as observed in this study is recommended. An analysis of the effect of colour "acclimation" on the retinal structure was not considered in the present study. However, the author acknowledges that research on retinal function is currently being conducted on fish exposed to both coloured background and coloured lights (Miriam Mass, School of Aquaculture University of Tasmania pers. comm.).

Some of the levels tested in the present study such as a temperature of 26 °C and a salinity of 5 ppt, resulted in 100% mortality. The temperature range for Chapter Three was selected from the temperature range that *H. abdominalis* experiences in their natural environment (8–24 °C). A temperature of 26 °C was selected in order to test whether it was possible to culture *H. abdominalis* at temperatures higher to the range used in commercial culture 16-19°C (Seahorse World Pty. Ltd.). Among the biological attributes of *H. abdominalis* are its large size (compared to most seahorse species), its elegant cirri and the variability in colouration patterns of their skin. However, being a temperate water species constitutes a disadvantage. The aquarium trade in seahorses is primarily focused on tropical species, such as *Hippocampus kuda, Hippocampus ingens* and *Hippocampus erectus*, which are more compatible with other tropical fish. Previous findings on the responses to higher temperatures of the non-tropical seahorse *Hippocampus whitei* have indicated improved growth in seahorses cultured at 26 °C (Wong and Benzie, 2003). This is not surprising given the geographical distribution of that species especially in the coastal areas (e.g. seagrass beds in Cliffton Gardens, Sydney) where the fish were collected for that study. The temperature study determined that *H. abdominalis* does not tolerate a temperature as high as 26 °C, which makes their use in the mainstream tropical aquarium fish market unfeasible. Future studies

could aim to determine the optimal temperature for the culture of this species by refining the temperatures tested in 0.5 °C increments from 23 °C to 25.5 °C. Nevertheless, the present results could benefit the commercial culture, especially for *H. abdominalis* rearing facilities which may experience temperature fluctuations over 23 °C.

The salinity range of 32 ppt to 5 ppt was selected in order to determinate the tolerance of *H. abdominalis* to low salinities. In Tasmania, commercial seahorse culture takes place in juvenile and broodstock tank systems serviced by a 75% daily water exchange from the Tamar River estuary rather than being supported by a recirculation system (Seahorse World Pty. Ltd. per. comm.). Based on the results from Chapter Five *H. abdominalis* culture facilities under similar circumstances to those of Seahorse World Pty. Ltd. could consider continuous monitoring of water salinity during rainfall runoff in order to maintain a water salinity higher than 10 ppt, which appears to affect negatively the condition of *H. abdominalis* after six weeks of exposure. Early juvenile seahorse survival improved by exposure to salinities as low as 10 ppt during the first ten days after birth, but even short-term exposure to 5 ppt was lethal. In addition, the salinity tolerance found in this study for *H. abdominalis* should be considered for future ventures such as seahorse cage culture *in situ* that has been relatively successful for tropical species in the Philippines as part of sustainable community programs (Vincent and Pajaro, 1997; Martin-Smith *et al.*, 2004). The risk of high mortalities due to high rainfall runoff must be considered during the design and orientation of the facilities, as cages located too close to the surface can potentially be exposed to freshwater in estuarine locations.

While in Chapter Three a temperature range higher than 17 °C was tested in order to assess its suitability for *H. abdominalis* culture, in Chapter Five the effects of a range of salinities lower than 32 ppt were compared as the seasonal reduction in salinity of the Tamar estuary is a recurrent problem. In both chapters the ranges tested were selected in response to the need of scientific knowledge posed by commercial seahorse culture. However, in future research, the examination of a range of temperatures below 17 °C, or a range of salinities higher than 32 ppt can contribute to the better understanding of the biology this species.

In Chapter Two the feeding rations used appeared to be in excess. However, the non-adjusted feeding ration possibly caused a confounding effect on the response of seahorses to different background colours. Ration was not increased proportionally as fish grew or died. Therefore, towards the end of the experiment, the fish cultured in tanks with high mortality were fed more *Artemia* per seahorse than fish cultured in tanks with no mortalities. This biased design motivated a review of the feeding protocol after the first experiment on temperature in Chapter Three. The amount of *Artemia* to be fed to each individual fish was calculated, providing only the amount of food correspondent to the exact number of remaining fish per tank each day. This protocol provided the 14% feed ration more accurately while minimizing confounding effects. This feeding strategy was complemented with the weekly weight measurements that provided a close reference to the seahorse's weight during the trials and contributed to the consistent feeding of a 14% body weight per day.

In Chapter Two, the measurement of the amount of food ingested was motivated by experimentation by Woods (2000). The technique was modified and the accuracy of the food ingestion measurement used in this study was assessed prior to the trials in Chapter Two; the use of the feeding strikes of seahorses proved an accurate measurement of *Artemia* ingestion during experiments. However, as the present study progressed it was noticed that there were no significant differences of *Artemia* ingestion among treatments. This lack of difference could be caused by the high variability of the data. However, during the second experiment on temperature in Chapter Three the *Artemia* ingestion data collected from the first observation week showed that fish cultured in 26 °C ingested low numbers of *Artemia* over the three days. Forty-eight hours after these observations all the seahorses cultured in 26 °C died. This suggests that the low level of *Artemia* ingestion may have been an indicator of stressful conditions or morbidity. A review of this technique is needed for future research in order to reduce variability.

At the end of the temperature experimentation (Chapter Three), a consideration was whether growth may have been influenced by a low density resulting from higher mortalities in some tanks, despite the ration levels being adjusted. An additional motivation for the experiments in Chapter Four was generated by the commercial culture of *H. abdominalis* which

experiences an increase in reproduction rates during the summer season. This production peak overwhelms the holding capacity of rearing systems (Seahorse World Pty. Ltd. per. comm.). The stocking density tested of 15 seahorses l^{-1} is double that used commercially and three times the stocking density used in most of the experiments in the present study (Chapter Two, Three, Five, and Six). During the preparation for the first experiment on stocking density, a number of tagging techniques were trialed to allow mortalities to be replaced by tagged fish to maintain density levels. Unfortunately, none of these attempts resulted in a reliable way to discriminate the replacements from the experimental fish. Consistent with the first experiment, there were no significant differences in treatments in survival. However, overall survival at the end of the second experiment with 21-day-old (50 %) was slightly lower than survival in the first experiment on younger seahorses (59 %). There were no differences in growth of seahorses cultured at a density of 45 seahorses 3 l^{-1} (15 seahorses l^{-1}) compared to seahorses cultured at a stocking density of 5 seahorses 3 l^{-1} (1.6 seahorses l^{-1}). Based on these results, it was concluded that a confounding effect caused by mortalities was unlikely to bias the result on growth of early juvenile seahorses during the experimentation with background colour, temperature, photoperiod and salinity.

During the long-term experiments (conducted over more than four weeks) in the present study there was a consistent pattern of mortality recorded in the first two weeks of the experiments. This mortality pattern has already been reported in early *H. abdominalis* (Woods, 2000) and in *H. comes* (Job *et al.*, 2006). This mortality has been attributed to food ingestion issues caused by changes in prey type preferences of seahorse as they grow (Sheng, 2006) or swimbladdder related problems (Woods, 2000). In the present study, among the overall mortalities recorded in the first two weeks, were observed seahorses with no swimbladder related issues. Also during experiments there was no change in prey type as juveniles were fed exclusively with enriched *Artemia*. The overall survival across long-term experiments in the present study was 52 ± 4 % (mean ± 1 S.E.) which is lower than the percentage survival of 80±% reported in studies conducted with late juveniles (Woods, 2003; Wong and Benzie, 2003). The aim of the present study was to contribute to the better understanding of the biology of *H. abdominalis* and to minimize mortality in early stages. However, more research is needed to determine the cause of this apparent inherent mortality in early stage seahorses.

The availability of fry in commercial seahorse culture is limited by k-selected reproductive strategy compared to the more r-selected reproductive strategy displayed by other marine teleosts. Based on the overall results of the present study, early juvenile culture can improve survival by using an adjusted feeding ration (based on mortality and weekly growth), a temperature of 20 °C, a salinity of 20 ppt, a stocking density of 10 seahorses l^{-1} and a photoperiod of 16:08 (L:D). This recommendation must be considered with some caution, as the combination of these factors was not tested simultaneously in the present study.

The range of photoperiods tested in the present study (Chapter Six) excluded a 12:12 (L:D) photoperiod as a reference treatment, as the use of 12-13 h of light has already proven to be effective in maintaining pot-bellied seahorse culture, commercially and during research, and did not fit the tank configuration available. Instead it was considered to be of greater interest to compare reduced/extended photoperiods at 8 h intervals against a treatment under continuous light which did not provide any benefit to early stage seahorses. At the end of the first experiment on photoperiod there was some concern regarding food limitation despite the adjusted feeding ration. The concern was motivated by the possibility that the nutritional value of the remaining *Artemia* in the tanks over the 24 h feeding cycle could be low due to evacuation of the enrichment media by the nauplius. This problem was addressed by increasing the food ration, although a more detailed examination is needed on the effect of using different photoperiods and feeding rations/frequencies. Ideally, it would be desirable to continuously supply nutritionally appropriate food in order to satisfy fish requirements. However, seahorse culture involves a live food preparation protocol which includes naupliar enrichment. Perhaps in future research on the use of green water culture during photoperiod experimentation may maintain an appropriate nutritional value of the live food in the tanks.

A potential extension of the research on photoperiod (Chapter 6) was the determination of associated melatonin hormone cycles to describe daily profiles, effect of daylength on these profiles and how it may relate to the growth observed. However, determination of the plasma melatonin production levels of the seahorses used in the experiments was not feasible because of the small sizes of the fish and the lack of methods dealing with whole body melatonin analysis. Instead, melatonin was analysed in adult fish as a surrogate (Chapter 7), the first analysis of any seahorse species. At the end of the analysis it was determined that

plasma melatonin is produced by pot-bellied seahorses and furthermore this species exhibits a diel profile of melatonin. The magnitude and patterns of melatonin production of the adults sampled suggest that the previously reported low locomotor/feeding activity in seahorses during the dark phase (Ouyang, 2005; Karina *et al.*, 2006; Sheng *et al.*, 2006) could be associated with the melatonin response of the fish to changes in photoperiod. However, there is a need to describe the effects of photoperiod on melatonin production to better understand the mechanisms behind diel patterns of activity/feeding. This will require experimentation with seahorses of a large enough size to obtain the minimal quantities of blood/plasma for the RIA or the examination of indirect techniques such as the water based measurement of melatonin proposed by James *et al.* (2004) which could be an option in the determination of melatonin profiles on small seahorses. Further work on the influence of photoperiod on melatonin could also contribute to our understanding of and ability to photo-manipulate reproductive cycles.

As previously discussed in the section on stocking density, the availability of space in seahorse culture depends partially on the availability of substrate as seahorses spend a large proportion of their life attached to substrate, particularly when not foraging for food or during the dark phase. This chapter successfully found that the substrate selection of newborns and 28-day-old seahorses discriminated against the small size substrate choices tested. This was evident during the first observations on 28-day-old seahorses when a proportion of seahorses preferred to hold onto the frame structure than to hold onto the choices provided. This preference for large diameter substrates was consistent in the subsequent observations on seahorses at both stages (newborns and 28-day-old), when in the absence of the frame structure most seahorses preferred the largest filament.

The present study was conducted under a multiple-choice design in which seahorses were given three different substrate diameters and three different mesh densities, from which they appeared to prefer the largest diameter and lowest mesh density. Future research is needed to determine their adaptability by examining their response when given a less preferred substrate (i. e. smaller) as the only choice. The seahorse industry requires more research on cost-effective husbandry solutions to seahorse culture requirements and the substrate preference of the pot-bellied seahorse is an important issue in order to provide an optimal

distribution of fish within culture tanks. Therefore, there is a need for the systematic examination of a wider range of materials, sizes, positions (i.e. vertical, horizontal) colours and designs, as seahorses do appear to show preferences.

The interaction of some of the factors tested in this study can be examined in further research. In the present study there was an indication that the effect of the interaction of a low salinity (15 ppt) with a high temperature (20 °C) produced poor growth. Further research on the effect of the interaction of different salinities with a range of temperatures is needed. Also, the effect of stocking density on the seahorse attachment preference can be tested by the combination of different substrate sizes or items distributed in a different density or in a different position. These substrate items could be made of a different colour or different substrate items could be allocated in tanks with different background colour.

A multi-factorial matrix to test a combination of factors is not feasible due to the requirement of a large number of fish of the same age. However, some combinations can be arranged to test two or more factors with the use of the results of this study in order to determine which would produce growth or survival improvements. For instance, the background colours tested did not produce a different response possibly due to the confounding factor of an uneven feeding ration. In a revised version of this research, these colours can be tested in combination with other factors such as: an adjusted feeding ration, a photoperiod of 16:08 (L:D), a temperature of 20 °C, a salinity of 20 ppt, a stocking density of 10 early juvenile seahorses l^{-1} and attachment substratum comprised of 3 mm nylon filaments separated 24 mm from each other.

Also the combination of different salinities with a range of temperatures can be tested using: an adjusted feeding ration, a photoperiod of 16:08 (L:D), a stocking density of 10 early juvenile seahorses l^{-1} and attachment substratum constituted by 3 mm nylon filaments separated 24 mm from each other.

The culture of early stages of the pot-bellied seahorse *H. abdominalis* presents different challenges compared to those of late juveniles. This thesis provides valuable information on the effect of a selection of factors on seahorse culture during the first two months of their

life; a stage not often approached due to the difficulties in maintaining an acceptable percentage of survival during experimentation.

From the results of this study it can be concluded that, early juveniles are capable of surviving and growing within a wide range of background (tank) colours. However, further research is needed to determine the response of seahorses to background colour with an adjusted feeding ration. The growth of early juvenile seahorse improves when using temperatures higher than 17 °C up to 23 °C. Seahorses also were able to grow and survive in salinities lower than the salinity of seawater from 32 ppt to 15 ppt at 17 °C, but the combination of a high temperature (20°C) with a low salinity (15 ppt) produced a negative effect on growth. The use of a temperature of 26 °C or a salinity of 5 ppt results in 100 % mortality within days. Juveniles are capable of surviving and growing at a density of 45 seahorses 3 l^{-1} (15 seahorses l^{-1}) with no significant differences compared to seahorses cultured at a stocking density of 5 seahorses 3 l^{-1} (1.6 seahorses l^{-1}). Based on these results, it appears unlikely that the level of mortality caused any density-related confounding effects biasing the results on growth of early juvenile seahorses during the experimentation with background colour, temperature, photoperiod and salinity. Seahorses in this study exhibited a preference for large substrate diameters and low mesh densities. Further research is needed to examine the relationship between stocking density and attachment substrate as it is likely to govern distribution of fish in the tank. The growth of juvenile seahorses improves when fish are cultured in extended photoperiods but not in continuous light, which possibly disrupts the rhythmicity of some physiological processes. The melatonin profile in adult fish showed that high levels of melatonin are produced during the scotophase and low melatonin levels are produced during the photophase. Melatonin is therefore likely to be involved in growth-related physiological processes in the fish. However, further research is needed to determine the effect of different photoperiods on the melatonin production of seahorses as this information can be used to optimise seahorse culture practices such as feeding and reproduction. This study presents a baseline for a future research and provides scientific information on seahorse husbandry which can be applied to commercial culture.

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Woods, C.M.C., 2003. Effect of stocking density and gender segregation in the seahorse *Hippocampus abdominalis*. Aquaculture 218, 167-176.

APENDIX ONE

10 Appendix one

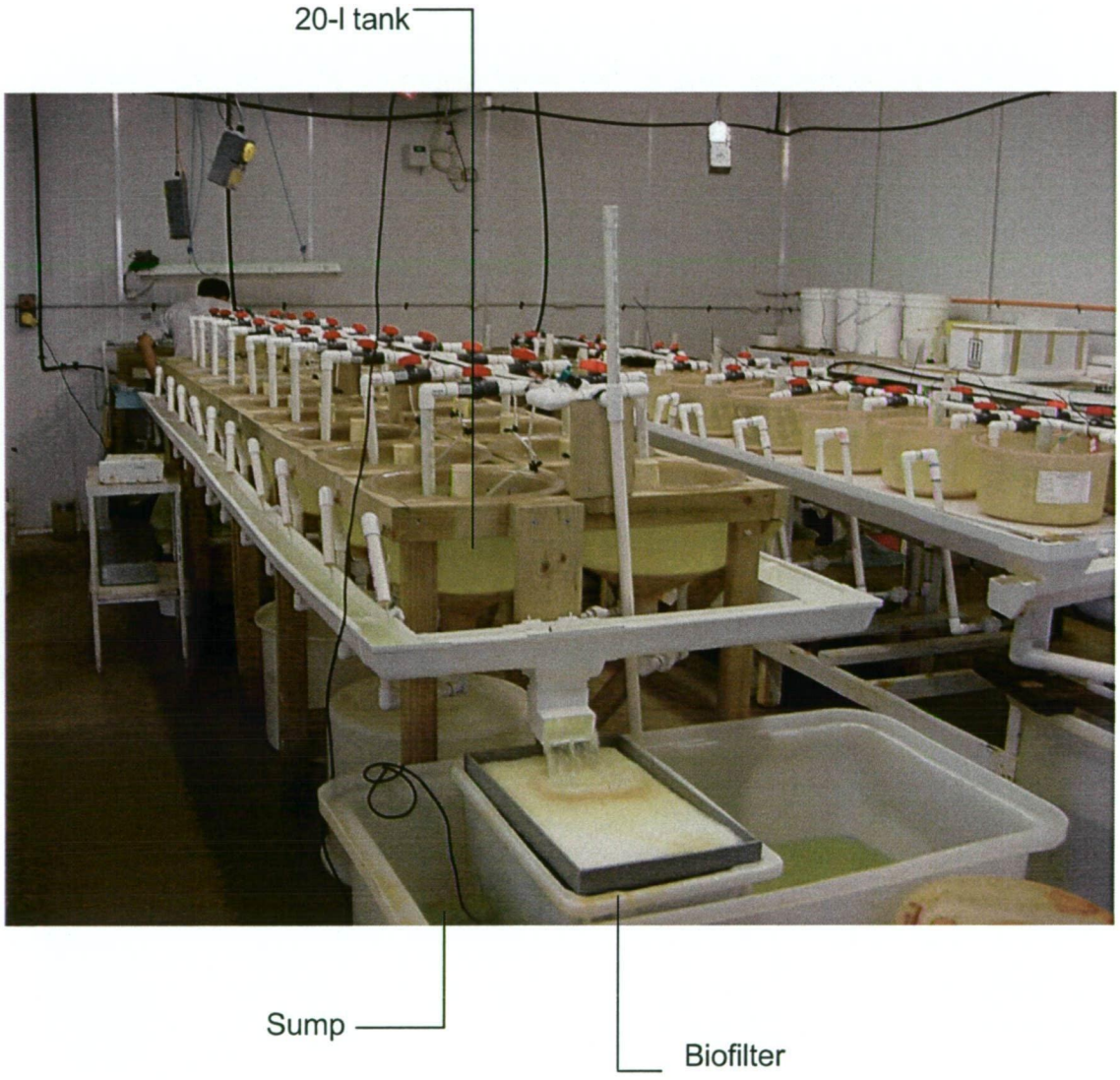

Diagram one. Recirculation system of 20 natural (fawn) coloured fibreglass 20-l holding tanks, where fish were allocated after 15-min temperature acclimation until the experiments started.

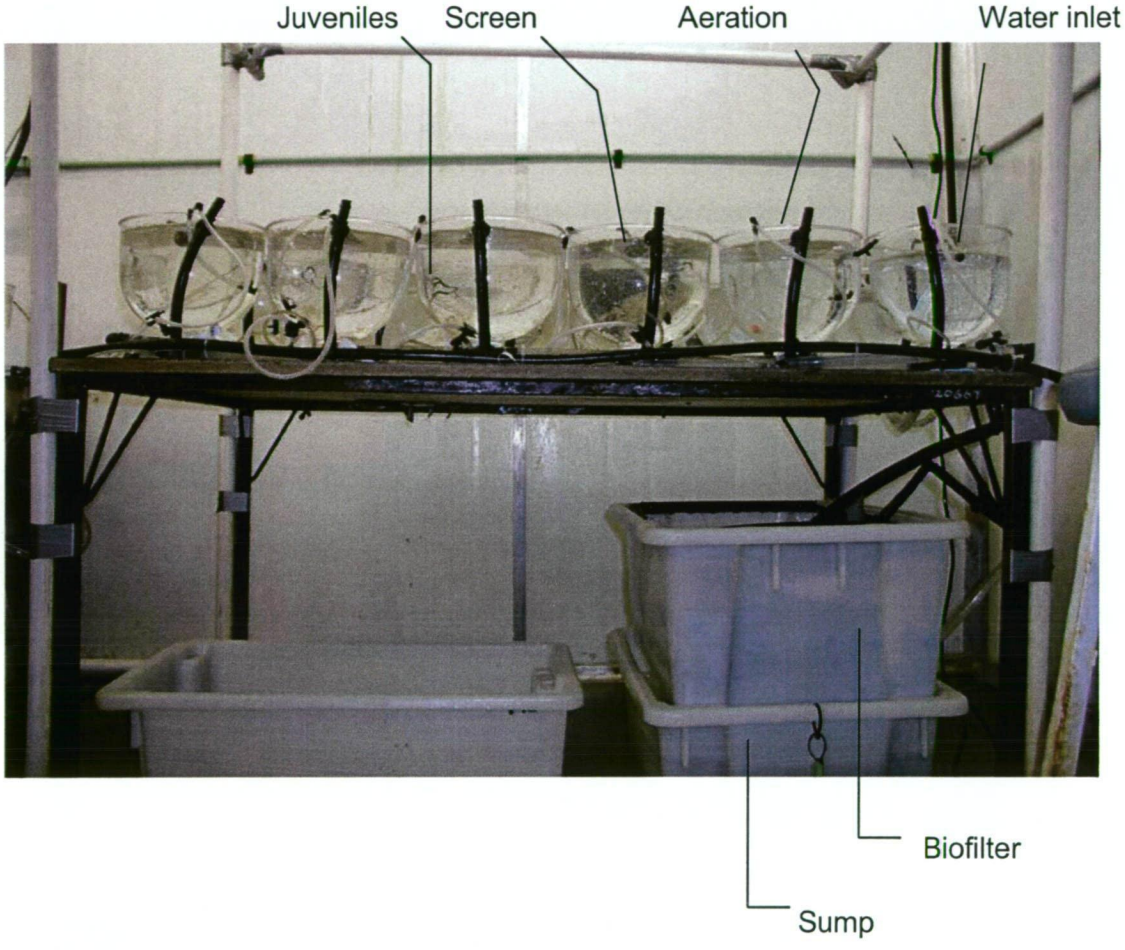

Diagram Two. Experimental tanks in a recirculation system were utilised in long-term experiments (background colour, stocking density, attachment-substrate preference and photoperiod).

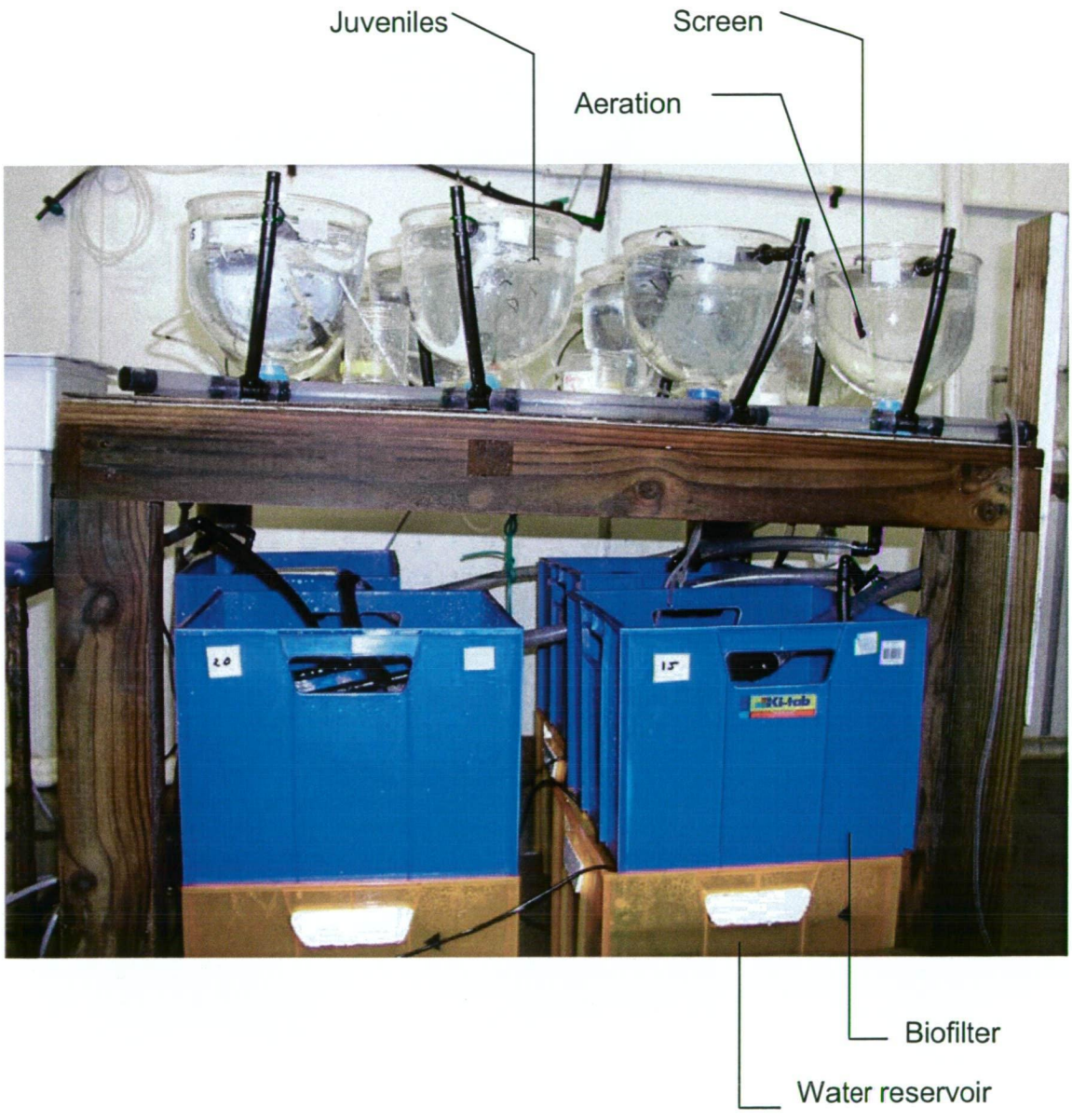

Diagram three. Experimental tanks in a recirculation system were utilised in long-term experiments (temperature, salinity). Each sub-system contained a separate bio-filter and heater (inside the water reservoir) to meet the water quality requirements and the desired temperatures.